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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The aim of this proposal is to develop a method to target novel cytotoxic agents specifically to sites of metastatic prostate cancer. Thapsigargin (TG) induces apoptosis in a proliferation independent manner in prostate cancer cells. This cytotoxicity, however, is not prostate cell type specific and TG could not be given systemically without significant toxicity. To achieve targeted cytotoxicity the TG analogs were converted to inactive prodrugs by coupling to a peptide carrier that is a substrate for the serine protease activity of Prostate-Specific Antigen (PSA). Since PSA is expressed in high levels only by normal and malignant prostate cells, this approach allows specific targeting of the killing ability of TG to prostate cancer cells. A series of amine containing TG analogs were synthesized and characterized for their ability to induce apoptosis in prostate cancer cell lines. The lead TG analog was chemically linked via a peptide bond to a previously identified PSA-specific peptide (i.e. 6 amino acids) to produce an inactive prodrug. This prodrug is hydrolyzed by PSA and is selectively toxic to PSA-producing prostate cancer cells in vitro and in vivo. On the basis of these studies, further clinical development of PSA-activated TG prodrug is warranted.

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INTRODUCTION:

Approximately forty thousand American men die annually from metastatic prostate cancer. Standard chemotherapeutic agents have been ineffective at significantly prolonging the survival of men with metastatic prostate cancer and these agents are typically associated with often severe, dose-limiting side effects. New agents are therefore urgently needed. While a large number of cytotoxic agents have been demonstrated to be effective in vitro, these agents are typically general cytotoxins that cannot be administered to patients without severe systemic toxicities. Therefore, what is required is a method to target the delivery of novel, effective cytotoxic agents specifically to sites of metastatic prostate cancer. Such an approach would result in increased concentration of drug within the tumor while avoiding significant systemic toxicity. One such novel agent that has been demonstrated in recent studies to induce apoptosis in a variety of cell types, including prostate cancers, is the natural product thapsigargin (TG). TG potently inhibits the Endoplasmic Reticulum Ca^{2+}-ATPase pump causing a sustained elevation of intracellular calcium that leads to induction of apoptosis in TG-treated cells. The cytotoxicity of TG, however, is not prostate cancer specific. In this proposal, a prostate cancer specific targeting strategy is outlined that will overcome this limitation. To achieve targeted cytotoxicity a potent TG analog will be converted to an inactive prodrug by coupling to a peptide carrier such that the analog can be efficiently converted back to an active killing agent only upon proteolysis by the serine protease activity of a unique prostate-specific protein, Prostate-Specific Antigen (PSA). Since PSA is expressed in high levels only by normal and malignant prostate cells and not in any significant amounts by other normal cell types, this approach should allow specific targeting of the killing ability of TG to prostate cancer cells. Therefore a series of primary amine containing TG analogs will be synthesized and characterized for their ability to induce apoptosis in prostate cancer cell lines and normal fibroblasts. Cytotoxic primary amine containing TG analogs will be chemically linked via a peptide bond to a previously identified PSA-specific peptide (i.e. 6 amino acids) to produce inactive prodrugs. Prodrugs in which the active TG analog can be efficiently released by the proteolytic activity of PSA will be tested for their potency and selectivity as PSA activated killing agents against PSA-producing, androgen independent human prostate cancer cells. The lead prodrug (i.e. the prodrug most efficiently and specifically hydrolyzed by PSA to release most active TG analog) will then be tested in vivo for activity in mice bearing PSA-producing human prostate cancers. These studies will serve to identify the best candidate prodrug that will subsequently tested in clinical trials as treatment for metastatic prostate cancer.

BODY:

The hypothesis of this proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, can be used to activate prodrugs specifically to cytotoxic metabolites at sites of metastatic prostate cancer. Originally, the specific cytotoxic agents to be targeted were proteasome inhibitors. The original plan of this New Investigator Award proposal was to develop prodrugs consisting of a proteasome inhibitor coupled to a peptide. The peptide was designed to be a specific substrate for the proteolytic activity of prostate-specific antigen (PSA). In this approach the inactive proteasome inhibitor/peptide prodrug could be given systemically without significant toxicity because PSA is enzymatically active within the blood due to complex formation with serum protease inhibitors. The extracellular fluid of prostate cancers contains large amounts (i.e. mg/ml) of enzymatically active PSA capable of releasing the active drug and inducing apoptosis of the surrounding cells.

The tasks of the original proposal were as follows:

Task 1: Synthesis of amine containing proteasome inhibitors and characterization of proteasome inhibition and cytotoxicity (1-12 months).

Task 2: Prodrug synthesis and determination of rates of PSA proteolysis of proteasome inhibitor prodrugs (12-24 months).

Task 3: To determine cytotoxicity and specificity of the prodrugs developed in Task 2 against PSA-producing and PSA-non-producing cells in vitro. (12-24 months)

Task 4: To determine the in vivo efficacy of the best (i.e. lead) PSA PI-prodrugs based upon comparison of the potency and specificity data of Task 3 (24-36 months).

For task 1, several amine containing proteasome inhibitors were synthesized (table 1) in collaboration with chemists at Cephalon, Inc. For these inhibitors a primary amine was incorporated into the structure of the inhibitor to allow for coupling to a peptide. These inhibitors were characterized for their ability to inhibit the proteasome in a broken cell proteasome assay, table 1. These compounds demonstrated potent inhibition of the proteasome at low nanomolar concentrations. In whole cell cytotoxicity assays, these compounds were far less potent with IC_{50} values for inhibition of cell growth in the micromolar range (i.e. 1000-fold less potent).

For task 2, the lead proteasome inhibitor was coupled to the PSA-specific peptide carrier Acetyl-His-Ser-Ser-Lys-Leu-Gln (Ac-HSSKLQ). This produced a compound that was still capable of potently inhibiting the proteasome in broken cell preparations with an IC_{50} value of 3 nM but demonstrated no cellular cytotoxicity at doses up to 50 μM. This prodrug was then incubated with enzymatically active PSA and assayed for hydrolysis by HPLC analysis. No demonstrable hydrolysis of the prodrug was observed (data not shown). In addition, HPLC analysis demonstrated that this proteasome inhibitor prodrug compound was unstable and rapidly degraded. The possible mechanism for this degradation is a reaction between the epsilon-primary amine of lysine with the boronic ester active group in the proteasome inhibitor. HPLC analysis also demonstrated instability of the uncoupled proteasome inhibitor by a potentially similar mechanism. Alternatively, the boronic ester group may not be stable in aqueous environment.

Originally, Cephalon Inc. had agreed to supply my laboratory with a series of modified proteasome inhibitors. This company also agreed to help with the synthesis of proteasome inhibitor-peptide prodrugs. However, on the basis of these preliminary unfavorable results, Cephalon Inc. did not wish to continue collaboration on this project. The company sited financial constraints and made a decision not to put any more of their already limited funds into the proteasome project.

These preliminary data obtained from work outlined in task 1 and 2 of the original application suggest that these proteasome inhibitors may not be ideal candidates for coupling to a peptide carrier due to their inherent instability and poor cell penetration. Instead of abandoning the project at this point, I chose to continue the work using an alternative cytotoxic agent. This decision was based on the previous findings in my laboratory suggesting that the defined PSA-specific peptide carrier could be used to effectively target a chemotherapeutic agent to sites of

PSA-producing prostate cancer. In this original work, a doxorubicin analog was coupled to the HSSKLQ peptide carrier to produce a prodrug that was stable and inactive in the absence of enzymatically active PSA (Appendix 1). However, in the presence of active PSA, the cytotoxic doxorubicin analog is released and cells underwent apoptosis. These preliminary studies with the doxorubicin prodrug provided the rationale for further development of this PSA-based targeting strategy. In work sponsored by this award, a PSA-doxorubicin prodrug was tested in vivo against a PSA-producing human prostate cancer xenograft. This prodrug was found to be non-toxic to the treated animals and significantly inhibited the rate of tumor growth. Doxorubicin has been tested previously as treatment for metastatic prostate cancer and, although partial responses were seen in some studies, this agent was not thought to be very effective therapy. Therefore, although doxorubicin may not be the preferred agent, other highly potent, novel cytotoxic agents could be employed in a similar PSA-targeted approach.

The original hypothesis of the proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, could be used to activate prodrugs specifically to liberate a cytotoxic agent at sites of metastatic prostate cancer. An example of one such cytotoxic agent is the natural plant product thapsigargin (TG) that has been demonstrated in recent studies to induce apoptosis in a variety of cell types, including prostate cancers, figure 1. TG is a potent inhibitor of the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase pump. TG has the unique ability to induce apoptosis in a proliferation independent manner. Therefore, it may be an ideal agent to treat slowly proliferating metastatic prostate cancer. TG would be difficult to administer systemically because its cytotoxicity is not prostate cancer specific and it is also able to kill G_0 arrested cells To achieve targeted cytotoxicity, TG analogs can also be converted to inactive prodrugs by coupling to a peptide carrier such that they can only be efficiently converted back to active killing agents only upon proteolysis by PSA.

Therefore, throughout the award the same initial approach was usedto develop a PSA-activated prodrug. Instead of the proteasome inhibitors, the natural plant product thapsigargin (TG) was substituted as the toxic "warhead" of the prodrug. The same task outline applied to develop a PSA-activated TG prodrug.

Task 1: Synthesis of amine containing TG analogs that could be coupled to a peptide carrier. Characterize inhibition of the SERCA pump and cytotoxicity of these TG analogs.

Result: A series of analogs were screened and a potent analog identified that could be coupled to PSA-specific peptide carrier. These results are summarized in attached publication (Appendix 1).

1. Jakobsen, C.M., **Denmeade, S.R.,** Isaacs, J.T., Gady, A.M., Olsen, C.E., Christensen, S.B. Design, Synthesis and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells. J. Med. Chem., 44: 4696-4703, 2001.

Task 2: Prodrug synthesis and determination of rates of PSA proteolysis of TG analog prodrugs.

Result: The lead TG analog (leucinyl-12-aminododecanoyl)-8-O-debutanoylthapsigargin (L12ADT) was coupled to the PSA-specific peptide carrier and rate of PSA hydrolysis and stability in human plasma determined. These results are summarized in attached publication (Appendix 2).

2. **Denmeade, S.R**., Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer. J NCI, in press, 2003.

Additional PSA prodrugs were also synthesized containing either a different PSA-selective peptide carrier or a different TG analogs. These analogs were also screened for PSA hydrolysis and stability in human plasma. A manuscript is in preparation detailing the results of these studies.

Task 3: To determine cytotoxicity and specificity of the prodrugs developed in Task 2 against PSA-producing and PSA-non-producing cells in vitro.

Result: PSA-activated prodrugs were selectively toxic to PSA-producing human prostate cancer cells in vitro when compared to PSA non-producing human prostate cancer cells. These results are also presented in the Appendix 2 manuscript.

Task 4: To determine the in vivo efficacy of the best (i.e. lead) PSA-activated TG prodrugs based upon comparison of the potency and specificity data of Task 3.

Result: TG prodrugs that were efficiently hydrolyzed by PSA, stable in human plasma, and selectively cytotoxic to PSA-producing human prostate cancer cells in vitro were tested for in vivo efficacy against PSA-producing and PSA non-producing human cancer xenografts. The lead prodrug with the peptide sequence His-Ser-Ser-Lys-Leu-Gln coupled to the L12ADT TG analog (i.e. HSSKLQ-L12ADT) was selectively toxic to PSA-producing LNCaP human prostate cancer xenografts while no antitumor effect against PSA non-producing SN12C human renal cancer xenografts was observed. These results are also presented in the Appendix 2 manuscript. Additional PSA-selective prodrugs were also tested in vivo against the LNCaP cell line and none of these prodrugs proved superior to the lead HSSKLQ-L12ADT prodrug. These results will be presented in a manuscript under preparation.

Additional tasks

Additional tasks were also completed that were outside the scope of the original New Investigator Award.

A. Pharmacokinetic analysis of lead prodrug was performed and demonstrated adequate plasma and tissue levels of lead HSSKLQ-L12ADT prodrug. Minimal (i.e. <0.5%) conversion to active L12ADT analog occurred in blood of treated mice. In contrast, adequate (i.e. 5-fold higher than in vitro IC_{50} concentrations) were observed in tumors from treated mice. These results are summarized in the Appendix 2 manuscript.

B. Characterized total in vitro and in vivo PSA production by a panel of human prostate cancer xenografts. In addition characterized amount of enzymatically active PSA produced by each of these xenografts to identify best models for prodrug testing. These studies resulted in two publications.

3. **Denmeade, S.R.**, Sokoll, L.J., Chan, D.W., Khan, S.R., Isaacs, J.T. Concentration of Enzymatically Active Prostate-Specific Antigen (PSA) in the Extracellular Fluid of Primary Human Prostate Cancers and Human Prostatic Cancer Xenograft Models. Prostate, 48:1-6, 2001.

4. **Denmeade, S.R.**, Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. Prostate, in press, 2002.

C. Participated in implementation and design of a series of studies to determine mechanism of TG-induced apoptosis. These studies were done in collaboration with Dr. Bertrand Tombal and Dr. John Isaacs at Johns Hopkins. These studies resulted in a number of publications over the period of this award.

5. Tombal, B., **Denmeade, S.R.**, and Isaacs, J.T. Assessment and Validation of a Microinjection Method for Kinetic Analysis of [Ca2+]i in Individual Cells Undergoing Apoptosis. Cell Calcium, 25:19-28, 1999.

6. **Denmeade, S.R.**, Lin, X., Tombal, B., and Isaacs, J.T. Inhibition of Caspase Activity Does Not Prevent the Signaling Phase of Apoptosis in Prostate Cancer Cells. Prostate 39:269-279, 1999.

7. Tombal, B., Weeraratna, A.T., **Denmeade, S.R.** and Isaacs, J.T. Thapsigargin Induces a Calmodulin/Calcineurin-dependent Apoptotic Cascade Responsible for the Death of Prostatic Cancer Cells. Prostate, 43:303-317, 2000.

8. Jackisch, C., Hahm, H., Tombal, B., McCloskey, D., Butash K., Davidson, N., and **Denmeade, S.R.** Delayed Micromolar Elevation in Intracellular Calcium Precedes Induction of Apoptosis in Thapsigargin-Treated Breast Cancer Cells. Clin Cancer Res, 6:2844-2850, 2000.

9. Tombal, B., **Denmeade, S.R.**, Gillis, J-M, Isaacs, J.T. A supramicromolar elevation of intracellular free calcium ([Ca2+]i) is consistently required to induce the execution phase of apoptosis. Cell Death and Differentiation 9:561-573, 2002.

D. Synthesized and characterized PSA-activated prodrugs of doxorubicin, 5-fluorouracil and taxanes in collaboration with Dr. Saeed Khan, a medicinal chemist and Assistant Professor at Johns Hopkins. These studies resulted in a number of publications and abstracts over the period of this award.

10. Khan S.R., **Denmeade, S.R.** In Vivo Activity of a PSA-Activated Doxorubicin Prodrug Against PSA-Producing Human Prostate Cancer Xenografts. Prostate, 45:80-83, 2000.

11. Mhaka, A., **Denmeade, S. R.,** Yao, W., Isaacs, J. T., Khan S. R. A 5-Fluorodeoxyuridine Prodrug as Targeted Therapy for Prostate Cancer. Bioorg. Medicinal Chemistry 12:2459-2461, 2002.

12. Mhaka, A. Yao, W., **Denmeade, S.R.**, Isaacs, J.T., Khan S.R. Synthesis and biological evaluation of novel chemotherapeutic prodrugs that require enzymatic activation by prostate specific antigen as treatment for prostate cancer. Proc. Am. Assoc. Cancer Res. 43:72, 2002.

13. Rosen, M. Gady. A. Buckley, T. **Denmeade, S.R.** Enzymatic activation of a modified proaerolysin toxin by prostate-specific antigen (PSA) as treatment for prostate cancer. Proc. Am. Assoc. Cancer Res. 43:92, 2002.

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized primary amine containing TG analogs that are potent cytotoxins
2. Demonstrated that a leucine containing TG analog (L-12ADT) can be coupled to a PSA-specific peptide and be hydrolyzed free from the peptide by enzymatically active PSA.
3. Demonstrated that this prodrug is relatively inactive against PSA non-producing cancer cells while ~ 30 fold enhancement of therapeutic effect occurs in the presence of PSA.
4. Demonstrated the in vivo efficacy of this HSSKLQ-L12ADT prodrug against PSA producing xenografts
5. Developed a method to determine pharmacokinetics of HSSKLQ-L12ADT using LC-MS.
6. Synthesized and tested additional amino acid containing TG analogs and determined enzyme kinetics.
7. Screened a series of additional PSA peptides substrates in attempt to optimize PSA sequence
8. Screened additional PSA analogs in vivo and identified a lead prodrug.
9. Characterized total in vitro and in vivo PSA production by a panel of human prostate cancer xenografts.
10. In addition characterized amount of enzymatically active PSA produced by each of these xenografts to identify best models for prodrug testing.
11. Participated in implementation and design of a series of studies to determine mechanism of TG-induced apoptosis.
12. Synthesized and characterized PSA-activated prodrugs of doxorubicin, 5-fluorouracil and taxanes in collaboration with Dr. Saeed Khan.

REPORTABLE OUTCOMES/BIBLIOGRAPHY:

Manuscripts, Abstracts;

1. Tombal, B., **Denmeade, S.R.**, and Isaacs, J.T. Assessment and Validation of a Microinjection Method for Kinetic Analysis of [Ca2+]i in Individual Cells Undergoing Apoptosis. Cell Calcium, 25:19-28, 1999.

2. **Denmeade, S.R.**, Lin, X., Tombal, B., and Isaacs, J.T. Inhibition of Caspase Activity Does Not Prevent the Signaling Phase of Apoptosis in Prostate Cancer Cells. Prostate 39:269-279, 1999.

3. **Denmeade, S.R.**, Jakobsen, C.M., Khan, S.R., Gady, A.M., Christensen, S.B., Isaacs, J.T. Enzymatic activation of a thapsigargin prodrug by prostate specific antigen (PSA) as treatment for metastatic prostate cancer. Proc. Am. Assoc. Cancer Research 41:46 #292, 2000.

4. Tombal, B., Weeraratna, A.T., **Denmeade, S.R**. and Isaacs, J.T. Thapsigargin Induces a Calmodulin/Calcineurin-dependent Apoptotic Cascade Responsible for the Death of Prostatic Cancer Cells. Prostate, 43:303-317, 2000.

5. Khan S.R., **Denmeade, S.R.** In Vivo Activity of a PSA-Activated Doxorubicin Prodrug Against PSA-Producing Human Prostate Cancer Xenografts. Prostate, 45:80-83, 2000.

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7. **Denmeade, S.R.**, Lovgren J., Khan, S.R., Lilja, H., Isaacs, J.T. Activation of Latent Protease Function of Pro-HK2, but not Pro-PSA, Involves Auto-Processing. Prostate, 48:122-126, 2001.

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9. Jakobsen, C.M., **Denmeade, S.R.**, Isaacs, J.T., Gady, A.M., Olsen, C.E., Christensen, S.B. Design, Synthesis and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells. J. Med. Chem., 44: 4696-4703, 2001.

10. Pinski, J., **Denmeade, S.R.**, Isaacs, J.T. Screening for agents which induce proliferation-independent apoptosis. Proc. Am. Assoc. Cancer Res. 42:482, 2001.

11. Tombal, B., **Denmeade, S.R.**, Gillis, J-M, Isaacs, J.T. A supramicromolar elevation of intracellular free calcium ([Ca2+]i) is consistently required to induce the execution phase of apoptosis. Cell Death and Differentiation 9:561-573, 2002.

12. Mhaka, A. Yao, W., **Denmeade, S.R.**, Isaacs, J.T., Khan S.R. Synthesis and biological evaluation of novel chemotherapeutic prodrugs that require enzymatic activation by prostate specific antigen as treatment for prostate cancer. Proc. Am. Assoc. Cancer Res. 43:72, 2002.

13. Rosen, M. Gady. A. Buckley, T. **Denmeade, S.R**. Enzymatic activation of a modified proaerolysin toxin by prostate-specific antigen (PSA) as treatment for prostate cancer. Proc. Am. Assoc. Cancer Res. 43:92, 2002.

14. Tombal, B., **Denmeade, S.R.**, Gillis, J-M, Isaacs, J.T. A supramicromolar elevation of intracellular free calcium ([Ca2+]i) is consistently required to induce the execution phase of apoptosis. Cell Death and Differentiation 9:561-573, 2002.

15. Mhaka, A., Denmeade, S. R., Yao, W., Isaacs, J. T., Khan S. R. A 5-Fluorodeoxyuridine Prodrug as Targeted Therapy for Prostate Cancer. Bioorg. Medicinal Chemistry 12:2459-2461, 2002.

16. **Denmeade, S.R.,** Litvinov, I., Sokoll, L.J., Lilja, H. Isaacs, J.T. Prostate-Specific Antigen (PSA) Protein Does Not Affect Growth of Prostate Cancer Cells In Vitro or Prostate Cancer Xenografts In Vivo. Prostate, In press, 2002.

17. **Denmeade, S.R.**, Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. Prostate, in press, 2002.

18. **Denmeade, S.R.**, Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer. J NCI, in press, 2002.

Presentations:

1. "PSA-Based Prodrug Therapy for Androgen Independent Prostate Cancer", CaPCURE Annual Meeting, Lake Tahoe, NV, 2001.
2. "PSA-Activated Prodrugs/Protoxins as Targeted Therapy for Prostate Cancer", AACR Special Meeting on Prostate Cancer, Naples, FL, 2001.
3. "Targeted Activation of Thapsigargin Prodrugs by Prostate-Specific Antigen (PSA) as Treatment for Prostate Cancer" Plenary Presentation, NCI National SPORE Meeting, Chantilly, VA, 2002.

Personnel receiving salary support:

Principal Investigator: Samuel R. Denmeade

Patents and Licenses applied for and/or issued;

1. "Tissue Specific Prodrug", Inventors: Isaacs, JT, Denmeade, SR, Christensen, SB, Lilja H. United States Patent Number 6,265,540 (filed 5/19/97 and 3/30/98). This patent refers to the PSA-specific peptides.

2. "Tissue Specific Prodrug", Inventors: Isaacs JT, Denmeade SR, Christensen SB, Lilja H. United States Patent Number 6,410,514 (filed 6/7/00 and 6/25/02). This patent refers to the creation of prodrugs activated by PSA.

3. "Tissue Specific Prodrug", Inventors: Isaacs JT, Denmeade SR, Christensen S. Patent filed 7/00. This invention refers to thapsigargin analogs for use as therapy for cancer.

CONCLUSIONS:

The original hypothesis of the proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, could be used to activate prodrugs specifically to liberate a cytotoxic agent at sites of metastatic prostate cancer. In the original proposal, I had intended to use proteasome inhibitors as the preferred cytotoxic agent. Preliminary studies demonstrated that this approach might not be feasible due to instability of the proteasome inhibitors and poor cell penetration. In addition, the pharmaceutical company that I was collaborating with decided not to continue this work, citing lack of funds.

Instead of abandoning the project at that early point, I decided to continue the project and use a different cytotoxic warhead by substituting a TG analog for the proteasome inhibitor. In doing so, I was building on the work with TG that I began as a post-doctoral fellow. TG represents an ideal treatment for slowly proliferating prostate cancers as it can induce apoptosis in a proliferation independent manner. To develop the TG-based prodrug I have followed the same task list and timetable as that outlined in the original proposal. For the renewal year I intend to complete tasks 3 and begin task 4 of the original proposal, substituting TG analogs for proteasome inhibitors as the cytotoxic agent that will be coupled to the PSA-specific peptide.

1. The preclinical data with the TG analog L12ADT (figure 2,3) demonstrate that it is a potent cytotoxin against prostate cancer cell lines. When coupled to the PSA-specific peptide, the prodrug HSSKLQ-L12ADT is readily hydrolyzed by PSA. This prodrug is relatively inactive in vitro in the absence of enzymatically active PSA in the media; however, when PSA is present the activity increases ~ 30 fold. In vivo, effective and specific antitumor activity is observed with the HSSKLQ-L12ADT at doses that are non-toxic to the host animal. Additional TG prodrugs have also been screened and demonstrate enhanced PSA hydrolysis. Additional PSA prodrugs that have improved hydrolysis kinetics and antitumor efficacy in vitro were synthesized and tested for in vivo against PSA-producing and non-producing xenograft. These prodrugs had similar in vivo antitumor efficacy as the HSSKLQ-L12ADT prodrug. On the basis of the studies outlined in Tasks 1-4, the HSSKLQ-L12ADT prodrug was selected as our lead prodrug that will be carried forward for toxicology studies and eventual clinical trial in patients with metastatic prostate cancer.

Appendices

Appendix 1:

Jakobsen CM, Denmeade SR, Isaacs JT, Gady A, Olsen C, Christensen SB. Design, synthesis and pharmacological evaluation of thapsigargin analogues for targeting apoptosis to prostatic cancer cells. J Med Chem 44:4696-4703, 2001.

Appendix 2:

Denmeade SR, Jakobsen CM, Janssen S, Khan SR, Lilja H, Christensen SB, Isaacs JT. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer. J NCI, in press 2002.

J. Med. Chem. **2001**, *44*, 4696−4703

Appendix 1

Design, Synthesis, and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells

Carsten M. Jakobsen,[†] Samuel R. Denmeade,[‡] John T. Isaacs,[‡] Alyssa Gady,[‡] Carl E. Olsen,[§] and Søren Brøgger Christensen*,[†]

Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark, Department of Experimental Therapeutics, Johns Hopkins Oncology Center, Bunting-Blaustein Cancer Research Building, 1650 Orleans Street, Baltimore, Maryland 21231, USA, and Chemistry Department, The Royal Veterinary and Agricultural University, 17 Bülowsvej, DK-1870 Frederiksberg, Denmark

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A series of thapsigargin (TG) analogues, containing an amino acid applicable for conjugation to a peptide specifically cleaved by prostate-specific antigen (PSA), has been prepared to develop the drug-moiety of prodrugs for treatment of prostatic cancer. The analogues were synthesized by converting TG into *O*-8-debutanoylthapsigargin (DBTG) and esterifying *O*-8 of DBTG with various amino acid linkers. The compounds were evaluated for their ability to elevate the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in TSU-Pr1 cells, their ability to inhibit the rabbit skeletal muscle SERCA pump, and their ability to induce apoptosis in TSU-Pr1 human prostatic cancer cells. The activity of analogues, in which DBTG were esterified with ω-amino acids [HOOC-$(CH_2)_n NH_2$, $n = 5-7$, 10, 11], increased with the linker length. Analogues with 3-[4-(L-leucinoylamino)phenyl]propanoyl, 6-(L-leucinoylamino)hexanoyl, and 12-(L-serinoylamino)dodecanoyl were considerably less active than TG, and analogues with 12-(L-alaninoylamino)dodecanoyl and 12-(L-phenylalaninoylamino)dodecanoyl were almost as active as TG. The 12-(L-leucinoylamino)dodecanoyl gave an analogue equipotent with TG, making this compound promising as the drug-moiety of a PSA sensitive prodrug of TG.

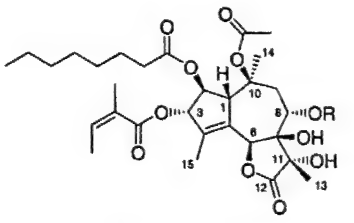
Introduction

Thapsigargin (TG, **1**) (Chart 1) is a sesquiterpene-γ-lactone isolated from seeds and roots of the umbelliferous plant, *Thapsia garganica* L.[1,2] TG selectively inhibits the ubiquitous sarcoplasmic and endoplasmic reticulum Ca^{2+}-dependent ATPases (SERCA's) with an apparent dissociation constant of 2.2 pM or less.[3,4] TG induced inhibition of the SERCA pump leads to depletion of the ER Ca^{2+} pool and a capacitance influx of extracellular Ca^{2+} resulting in a sustained elevation (i.e., 200−400 nM) of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$).[5] This sustained elevation of $[Ca^{2+}]_i$ subsequently leads to DNA fragmentation and programmed cell death (apoptosis) of treated cells.

TG induces apoptosis in rat (AT-3) and human (TSU-Pr1, PC-3, DU-145) androgen-independent prostatic cancer cell lines with LC_{50} values for cell death in the 10−100 nM range.[5] This TG induced apoptosis does not require the cells to be in proliferative cell cycle but can be induced in primary human prostatic cancer cell cultures in which about 98% of the cells are out of cycle in G_0.[6] These studies have identified the SERCA pump as a new therapeutic target for activating apoptosis of androgen-independent prostatic cancer cells.

TG's ability to kill proliferatively quiescent G_0 cells by inhibiting the ubiquitous SERCA's means that it will be difficult to administer TG systemically as a therapeutic agent without significant host toxicity. One approach to specifically target TG cytotoxicity to prostatic cancer cells is to take advantage of the unique secretion of prostate-specific antigen (PSA) by these cells. PSA is a serine protease with chymotrypsin-like substrate specificity that is enzymatically active only in the extracellular fluid of prostatic cancer cells, whereas it is enzymatically inactivated in blood serum.[7−9] Previously a highly specific and efficient PSA substrate with the sequence His−Ser−Ser−Lys−Leu−Gln-(HSSKLQ) was identified.[9] If TG is converted to *O*-8-debutanoylthapsigargin (DBTG, **2**) (Chart 1) and DBTG is esterified in the *O*-8 position with an amino carboxylic acid linker, the resulting TG analogue can be coupled to the C-terminal glutamine (Q) of this peptide to form a peptide bond that is hydrolyzable by enzymatically active PSA (Figure 1). Such a TG analogue prodrug will be cleaved only in the extracellular fluid of PSA-

Chart 1[a]



TG (**1**), R = $CO(CH_2)_2CH_3$

DBTG (**2**), R = H

[a] Structures of thapsigargin (TG, **1**) and *O*-8-debutanoylthapsigargin (DBTG, **2**).

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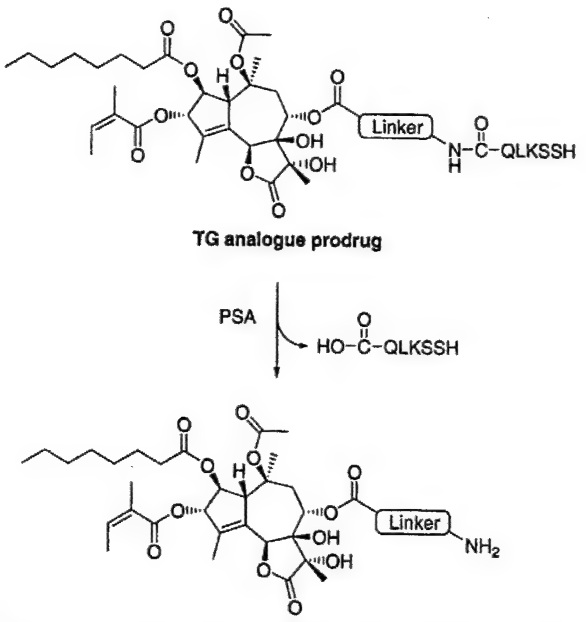


Figure 1. Prostate-specific antigen (PSA) releases the active TG analogue from the TG analogue prodrug by hydrolysis of the peptide promoiety (HSSKLQ).

secreting prostatic cancer cells thus specifically targeting the TG analogue cytotoxicity to prostatic cancer cells.

In a previous study,[10] we reported on the synthesis and apoptotic activity of a series of *O*-8 substituted aromatic amine TG analogues (**3c**,**e**,**f** and **4a**−**e**) (Chart 2). Analogues **4c**−**e** were the most potent, being 1.5, 1.7, and 1.7 times less potent inhibitors of the rabbit skeletal muscle SERCA pump than TG, respectively, and 9, 4, and 8 times less cytotoxic against TSU-Pr1 human prostatic cancer cells, respectively.

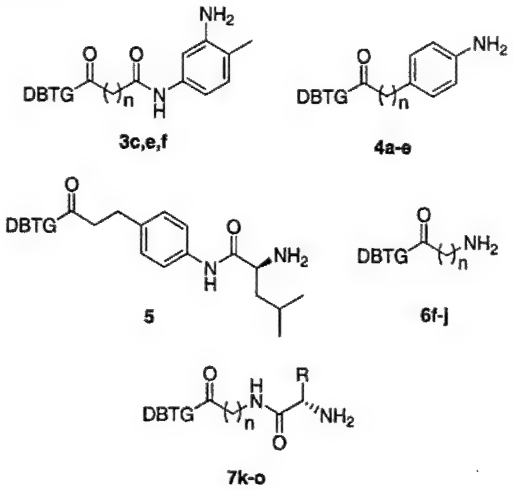
The goal of this paper was to investigate the structure−activity relationship of TG analogues, in which the *O*-8 butyric acid has been substituted with an ω-amino acid (analogues **6f**−**j**) (Chart 2) or an α-amino acid conjugated ω-amino acid (analogues **5** and **7k**-**o**) (Chart 2).

All target compounds were evaluated for their ability to elevate $[Ca^{2+}]_i$ in TSU-Pr1 cells, their ability to inhibit the rabbit skeletal muscle SERCA pump, and their ability to kill TSU-Pr1 human prostatic cancer cells.

Chemistry

DBTG (**2**) was prepared from TG (**1**) by selective trans-esterification of the *O*-8-butanoyl ester in methanol using triethylamine as catalyst.[11,12] Analogue **5** was synthesized as shown in Scheme 1. 4-Amino-*trans*-cinnamic acid hydrochloride was hydrogenated and the carboxyl group protected as the methyl ester to give **8**. N_α-Boc-L-leucine was coupled[13] to the aromatic amine to give **9**. Deprotection of the carboxylic acid gave **10**, coupling to **2** gave **11**, and deprotection of the amine gave analogue **5**. Analogues **6f**−**j** were synthesized as outlined in Scheme 2. Coupling of the *N*-Boc protected aliphatic amino acids **12f**−**j** with **2** gave **13f**−**j**, and deprotection of the amino group gave **6f**−**j**. Analogues **7k**−**m** were synthesized as shown in Scheme 3. N_α-Boc-L-leucine was coupled to methyl esters **14f**,**j** to give **15k**,**l**, and N_α-Boc-L-alanine was coupled to methyl ester **14j** to give **15m**. Deprotection of the carboxylic acid gave **16k**−**m**, coupling to **2** gave **17k**−**m**, and deprotection of the amine gave **7k**−**m**. Analogues **7n**,**o** were synthesized as outlined in Scheme 4. N_α-Boc-L-serine and N_α-Boc-L-phenylalanine were coupled to analogue **6j** to give **18n**,**o**, and deprotection of the amine gave **7n**,**o**.

Chart 2[a]



a: n = 0 | **i**: n = 10
b: n = 1 | **j**: n = 11
c: n = 2 | **k**: n = 5, R = $CH_2(CH_3)_2$
d: n = 2, *trans* C=C | **l**: n = 11, R = $CH_2(CH_3)_2$
e: n = 3 | **m**: n = 11, R = CH_3
f: n = 5 | **n**: n = 11, R = CH_2OH
g: n = 6 | **o**: n = 11, R = CH_2Ph
h: n = 7

[a] TG analogues with amine-containing linkers esterified to the *O*-8 hydroxyl of **2**.

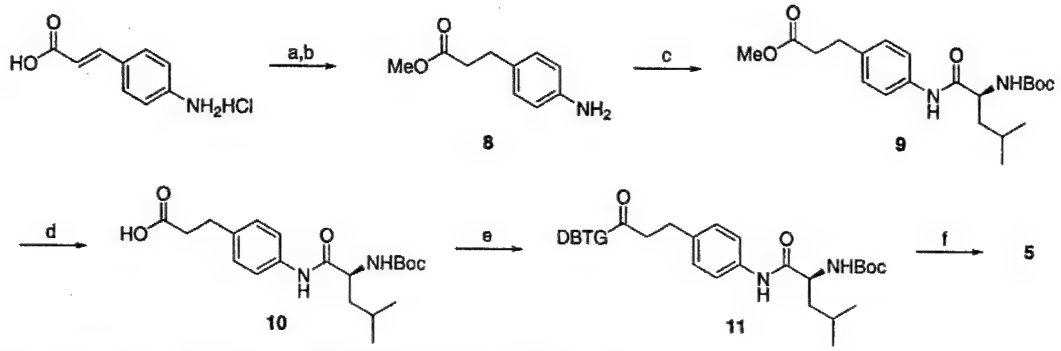
Pharmacology

SERCA containing microsomes were isolated from rabbit skeletal muscle by differential centrifugation of the muscle homogenate.[14,15]

The SERCA activity was measured with a coupled enzyme assay as the rate of ATP hydrolysis.[16,17] The activity at each dose (nmol/mg of SR protein) of TG analogue was expressed as percentage of the uninhibited control activity and was determined in triplicate. Inhibition curves were corrected for Ca^{2+}-independent (TG insensitive) ATPase activity by subtracting the residual activity at high inhibitor concentrations, which typically represented 10% of the total activity. The amount of TG analogue required to inhibit 50% of the maximal Ca^{2+}-dependent ATPase activity in 1 mg of SR protein was expressed as ID_{50} values, and was determined by 4-parameter curve-fitting (Table 1).

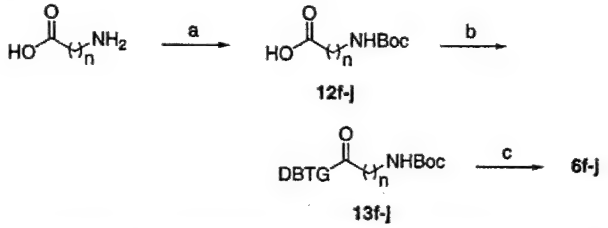
The apoptotic activity of each TG analogue against TSU-Pr1 human prostatic cancer cells was determined as previously described.[18] The apoptotic activity was expressed as the concentration of analogue LC_{50} (μM) capable of inducing 50% loss of clonogenic survival as compared to untreated controls (Table 1).

Scheme 1[a]



[a] Reagents: (a) Pd/C, H_2, 2-metoxyethanol; (b) MeOH, $SOCl_2$; (c) N_α-*tert*-butoxycarbonyl-L-leucine, hexachloroacetone, Ph_3P, pyridine, THF; (d) 2 M NaOH (aq), MeOH; (e) **2**, DCC, DMAP, CH_2Cl_2; (f) TFA, CH_2Cl_2.

Scheme 2[a]



[a] Reagents: (a) 5 M NaOH (aq), $(Boc)_2O$, *tert*-BuOH; (b) **2**, DCC, DMAP, CH_2Cl_2; (c) TFA, CH_2Cl_2.

The increase in cytosolic calcium concentration ($[Ca^{2+}]_i$) in TSU-Pr1 cells induced by TG analogues at effective cytotoxic concentrations was determined as previously described (Table 2).[10,19]

Results and Discussion

Previous published results concerning PSA activated doxorubicin prodrugs[20] promoted us to conjugate analogue **4c** with L-leucine to give analogue **5**. The conjugated analogue showed decreased SERCA inhibition and apoptotic activity (Table 1). The decreased activity of **5** was attributed to a decreased lipophilicity due to protonization of the α-amine at physiological pH. Previously, a positive correlation between lipophilicity and histamine-releasing activity was found in a series of *O*-2 and *O*-8 substituted TG analogues.[21]

The previously prepared aromatic analogues **3c**,**e**,**f** were 328, 81, and 4.4 times less potent SERCA inhibitors than TG, respectively, and >800, 533, and 103 times less cytotoxic, respectively.[10] Apparently, the potency also in this case increases with increasing lipophilicity. A similar structure–activity relationship was found in the series of aliphatic analogues **6f–j** (Table 1).

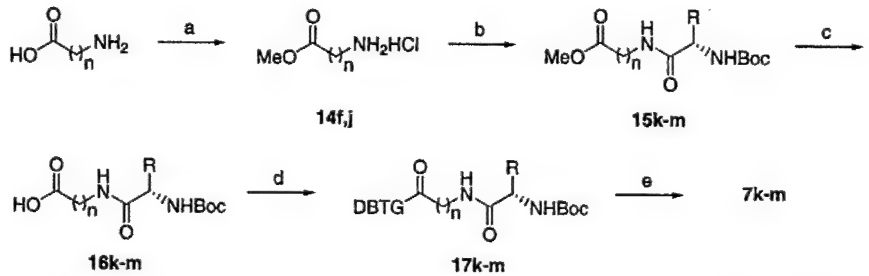
Table 1. ID_{50} (nmol/mg of SR protein) Values for Inhibiting 50% of Maximal Rabbit Skeletal Muscle SERCA Activity and LC_{50} (μM) Values for 50% Loss of Clonogenic Survival of Human Prostate Cancer TSU-Pr1 Cells[a]

compd	ID_{50} (nmol/mg SR protein)	activity relative to TG[b]	LC_{50} (μM)	activity relative to TG[c]
TG	13.4 ± 1.4	1	0.03 ± 0.004	1
5	466 ± 22	35	0.88 ± 0.04	29
6f	1332 ± 83	99	>20	>667
6g	1206 ± 57	90	10.92 ± 2.28	364
6h	223 ± 15	17	3.85 ± 0.21	128
6i	40 ± 3	3.0	0.75 ± 0.03	25
6j	35 ± 4	2.6	1.16 ± 0.16	39
7k	3842 ± 315	287	>20	>667
7l	45 ± 3	3.4	0.03 ± 0.01	1.0
7m	16.5 ± 1.6	1.2	0.28 ± 0.06	9.3
7n	10.3 ± 0.5	0.8	0.89 ± 0.04	30
7o	n.d.	-	0.21 ± 0.02	7.0

[a] Results expressed as mean ± standard deviation of triplicate measurements. [b] ID_{50} analogue/ID_{50} TG. [c] LC_{50} analogue/LC_{50} TG.

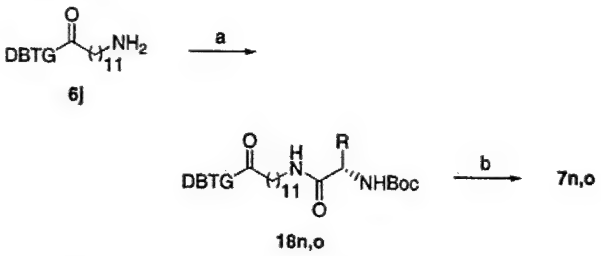
Conjugation of **6f** with L-leucine to give **7k** decreased the SERCA inhibitory activity, and the apoptotic activity was still poor (Table 1). In contrast, conjugation of **6j** with L-leucine to give **7l** only marginally changed the SERCA inhibition, and surprisingly increased the apoptotic activity affording an analogue equipotent with TG. Replacement of L-leucine in **7l** with L-alanine and L-phenylalanine (**7m**,**o**, respectively) only to a limited extent influenced the activity, whereas introduction of the more hydrophilic L-serine (**7n**) afforded a less apoptotic analogue.

Scheme 3[a]



[a] Reagents: (a) MeOH, $SOCl_2$; (b) N_α-*tert*-butoxycarbonyl-L-leucine or N_α-*tert*-butoxycarbonyl-L-alanine, DIPEA, DCC, CH_2Cl_2; (c) 2 M NaOH (aq), MeOH; (d) **2**, DCC, DMAP, CH_2Cl_2; (e) TFA, CH_2Cl_2.

Scheme 4[a]



[a] Reagents: (a) N_α-*tert*-butoxycarbonyl-L-serine or N_α-*tert*-butoxycarbonyl-L-phenylalanine, DCC, HOBT, DMF; (b) TFA, CH_2Cl_2.

Table 2. Increase in Cytosolic Ca^{2+} Concentration ($[Ca^{2+}]_i$) in TSU-Pr1 Cells at Effective Cytotoxic Concentrations[a]

compd	1000 nM $[Ca^{2+}]_i$ (nM)	100 nM $[Ca^{2+}]_i$ (nM)
5	451 ± 54	91 ± 16
6f	284 ± 51	NE[b]
6g	209 ± 6	NE[b]
6h	217 ± 20	NE[b]
6i	420 ± 3	242 ± 7
6j	369 ± 2	235 ± 12
7k	84 ± 7	40 ± 3
7l	414 ± 44	173 ± 28
7m	410 ± 45	338 ± 10
7n	348 ± 20	93 ± 10
7o	446 ± 33	406 ± 15

[a] $[Ca^{2+}]_i$ was monitored for 20 min. Baseline $[Ca^{2+}]_i$ was 35 ± 4 nM. Results are expressed as the mean ± standard error of triplicate measurements. [b] NE = no significant elevation of $[Ca^{2+}]_i$ above baseline level.

The ability of the analogues to elevate $[Ca^{2+}]_i$ in TSU-Pr1 cells followed the relative SERCA inhibitory and apoptotic activities (Table 2).

In conclusion, a series of TG analogues containing an α-amino acid applicable for conjugation to PSA-specific peptides has been prepared. In general, the potency follows the relative lipophilicity of the analogues. The high activity of **7l** makes this analogue especially interesting as a drug moiety in a prodrug of TG.

Experimental Section

Chemistry. TG (**1**) was isolated from the seeds of *Thapsia garganica* L. as previously described.[1] Reagents and precursors were supplied by Aldrich and were used without further purification. Thin-layer chromatography was done using precoated aluminum sheets with Silica gel 60 F_{254} or RP-18 F_{254} (Merck). Compounds were visualized by inspection under UV (λ = 254 nm) or after spraying with naphthoresorcinol solution (0.2% w/v in ethanol diluted 1:1 with 2 M H_2SO_4) or ninhydrin solution (0.05% w/v in ethanol) followed by heating. Normal phase column chromatography (NPCC) was done with Silica gel 60, 40−63 μm (Merck) using mixtures of EtOAc−toluene−AcOH, 20:10:0.3 (A), heptane−acetone−AcOH, 7:3:0.1 (B), or toluene−acetone, 19:1 (C), and 1:1 (D). Reverse phase column chromatography (RPCC) was done with LiChroprep RP-18, 40−63 μm (Merck) using mixtures of MeOH-water, 4:1 (E), 5:1 (F), 6:1 (G), 7:1 (H), and 9:1 (I) or MeOH−water−AcOH, 9:1:0.1 (J). Melting points were measured with capillary tubes in an oil bath and were corrected. ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a GEMINI 2000 BB, 300 MHz spectrometer. Chemical shift values (δ) are expressed in ppm relative to tetramethylsilane as internal standard. The following abbreviations are used for multiplicity of NMR signals: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet. NMR signals corresponding to exchangeable protons are omitted. Signals from α-amino acids are assigned with Greek letters α, β, and γ. Signals originating from the TG nucleus of all the *O*-8 acylated compounds were in the following range: ^1H NMR ($CDCl_3$) δ 0.86−0.87 (t, J = 7−8 Hz, 3H, octanoyl H-8), 1.26−1.28 (m, 8H, octanoyl H-4 to H-7), 1.37−1.44 (br s, 3H, H-14), 1.39−1.50 (s, 3H, H-13), 1.58−1.60 (m, 2H, octanoyl H-3), 1.83−1.87 (br s, 3H, H-15), 1.88−1.90 (s, 3H, acetyl H-2), 1.90−1.92 (m, 3H, angeloyl C-2 CH_3), 1.99−2.00 (d, J = 7−8 Hz, 3H, angeloyl H-4), 2.28−2.30 (m, 2H, octanoyl H-2), 2.28−2.42 (dd, J = 3−4 and 13−15 Hz, 1H, H-9′), 2.89−3.03 (dd, J = 3−4 and 13−15 Hz, 1H, H-9), 4.18−4.33 (br s, 1H, H-1), 5.46−5.51 (m, 1H, H-2), 5.61−5.71 (m, 3H, H-3, H-6 and H-8), 6.10−6.12 (q, J = 7−8 Hz, 1H, angeloyl H-3). Some times H-8 appeared at a slightly lower ppm value than H-3 and H-6 and some times H-9′ was overlapped by signals from the acyl residues. ^{13}C NMR ($CDCl_3$) (labeled assignments are interchangeable) δ 12.8−13.4 (C-15), 14.1−14.4 (octanoyl C-8), 15.7−16.1 (C-13), 15.8−16.3 (angeloyl C-4), 19.6−20.8 (angeloyl C-2 CH_3), 22.2−22.8 (acetyl C-2), 22.5−22.8 (C-14), 23.6−24.4 (octanoyl C-7), 24.3−25.2 (octanoyl C-3), 29.0−29.5 (octanoyl C-4 to C-6), 34.6−36.1 (octanoyl C-2), 38.3−40.7 (C-9), 57.5−59.4 (C-1), 66.2−66.5 (C-8)[a], 77.0−77.8 (C-6)[a], 77.9−78.8 (C-2)[a], 78.5−78.9 (C-7)[b], 78.6−79.0 (C-11)[b], 84.3−85.7 (C-3)[a], 84.7−85.7 (C-10), 127.7−127.9 (angeloyl C-2), 130.1−131.2 (C-4)[c], 138.8−139.2 (angeloyl C-3), 141.1−142.1 (C-5)[c], 165.1−167.4 (C=O, angeloyl), 170.9−171.8 (C=O, acetyl), 172.9−173.1 (C=O, octanoyl), 174.5−177.6 (C=O, C-12). The identity of target compounds was confirmed with NMR and HRMS. The target compounds were pure according to TLC, and their NMR spectra showed no foreign signals other than minor solvent residuals.

8-*O*-Debutanoylthapsigargin (2). Triethylamine (2.5 mL) was added to a solution of **1** (0.80 mmol) in dry MeOH (50 mL) at room temperature. After 6 h at room temperature, the mixture was concentrated in vacuo. The residue was concentrated two times from toluene (50 mL) in vacuo to give **2** (yield 100%) as a white amorphous solid: ^1H NMR ($CDCl_3$) δ 0.87 (t, J = 7.0 Hz, 3H, octanoyl H-8), 1.28 (br s, 8H, octanoyl H-4 to H-7), 1.44 (s, 3H, H-14), 1.49 (s, 3H, H-13), 1.60 (m, 2H, octanoyl H-3), 1.84 (s, 3H, H-15), 1.90 (m, 6H, acetyl H-2 and angeloyl C-2 CH_3), 1.99 (d, J = 7.0 Hz, 3H, angeloyl H-4), 2.29 (m, 2H, octanoyl H-2), 2.47 (dd, J = 3.3 and 14.1 Hz, 1H, H-9′), 2.84 (d, J = 14.1 Hz, 1H, H-9), 3.57 (br s, 1H, H-8), 4.36 (s, 1H, H-1), 5.45 (m, 1H, H-2), 5.69 (s, 1H, H-3), 5.80 (s, 1H, H-6), 6.12 (q, J = 7.2 Hz, 1H, angeloyl H-3).

3-(4-Aminophenyl)propionic Acid Methyl Ester (8). Triethylamine (10.0 mmol) was added dropwise to a suspension of 4-amino-*trans*-cinnamic acid hydrochloride (10.0 mmol) in 2-methoxyethanol (12 mL) at room temperature. The suspension was filtered, and Pd-C 10% (120 mg) was added to the filtrate. The mixture was hydrogenated (4 atm) for 3 h at room temperature. The mixture was filtered through a column of Celite, and the filtrate was concentrated in vacuo to give 3-(4-aminophenyl)propionic acid (1.7 g) as a yellowish crystalline solid. This compound was esterified without further purification. Thionyl chloride (2.5 mL) was added dropwise to dry MeOH (10 mL) at −10 °C, and the solution was left for 10 min. 3-(4-Aminophenyl)propionic acid (1.7 g) was added to the solution, and the mixture was left overnight at room temperature. The solvent was removed in vacuo, and the residue was dissolved in EtOAc (200 mL). The solution was washed with 5% $NaHCO_3$ (200 mL), 10% NaCl (100 mL), and water (100 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **8** (yield 80%) as a crystalline yellow solid: ^1H NMR ($CDCl_3$) δ 2.55 (t, J = 7.0 Hz, 2H, H-2), 2.85 (t, J = 7.0 Hz, 2H, H-3), 3.65 (s, 3H, OCH_3), 6.60 (d, J = 9.0 Hz, 2H, Ar H-3 and H-5), 6.95 (d, J = 9.0 Hz, 2H, Ar H-2 and H-6).

3-(4-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]phenyl)-propanoic Acid Methyl Ester (9). A solution of triphenylphosphine (1.00 mmol) in dry THF (1.0 mL) was under argon dropwise added to a solution of N_α-*tert*-butoxycarbonyl-L-leucine (1.00 mmol) and hexachloroacetone (0.50 mmol) in dry THF (2.0 mL) at −78 °C, and the solution was left for 30 min. A solution of compound **8** (1.00 mmol) in dry THF (1.0 mL)

and a solution of pyridine (6.00 mmol) in dry THF (6.0 mL) was added to the reaction mixture at −78 °C, and the mixture was left at room temperature for 1 h and filtered. The filtrate was concentrated in vacuo, and the residue was dissolved in CH_2Cl_2 (100 mL). The solution was washed twice with 1 M HCl (50 mL), twice with 5% $NaHCO_3$ (50 mL), and twice with 10% NaCl (50 mL) and concentrated in vacuo. Purification of the residue by NPCC (eluent A) afforded **9** (50%) as a yellowish crystalline solid: ^{1}H NMR ($CDCl_3$) δ 0.95 (m, 6H, Leu CH_3 and CH'_3), 1.43 (s, 9H, Boc CH_3), 1.62 (m, 1H, γ-H), 1.72 (m, 2H, β-H), 2.58 (t, J = 7.8 Hz, 2H, H-2), 2.88 (t, J = 7.8 Hz, 2H, H-3), 3.66 (s, 3H, OCH_3), 4.35 (m, 1H, α-H), 7.07 (d, J = 8.1 Hz, 2H, Ar H-2 and H-2′), 7.41 (d, J = 8.1 Hz, 2H, Ar H-3 and H-3′); ^{13}C NMR ($CDCl_3$) δ 22.3 (Leu CH_3), 23.6 (Leu CH'_3), 25.2 (γ-C), 28.5 (Boc CH_3), 31.0 (C-3), 36.5 (C-2), 41.7 (β-C), 52.1 (OCH_3), 54.5 (α-C), 81.2 (Boc *tert*-C), 120.3 (Ar C-3 and C-5), 129.0 (Ar C-2 and C-6), 132.7 (Ar C-1), 136.7 (Ar C-4), 156.4 (C=O, carbamate), 171.2 (C=O, C-1), 173.4 (C=O, amide).

3-(4-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]phenyl)-propanoic Acid (10). 2 M NaOH (10 mL) was added at room temperature to a solution of **9** (4.89 mmol) in MeOH (65 mL), and the mixture was left for 10 min at room temperature. The MeOH was removed in vacuo, and the aqueous residue was acidified to pH 2 with 2 M H_2SO_4. The aqueous solution was extracted twice with EtOAc (60 mL), and the combined organic phases were washed with 10% w/v NaCl (60 mL) and water (60 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **10** (99%) as a yellowish crystalline solid: mp 64.5−66.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.3 Hz, 3H, Leu CH_3), 0.96 (d, J = 6.3 Hz, 3H, Leu CH'_3), 1.38 (s, 9H, Boc CH_3), 1.66 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.63 (t, J = 7.2 Hz, 2H, H-2), 2.89 (t, J = 7.2 Hz, 2H, H-3), 4.48 (m, 1H, α-H), 7.05 (d, J = 8.0 Hz, 2H, Ar H-2 and H-6), 7.43 (d, J = 8.0 Hz, 2H, Ar H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ 21.9 (Leu CH_3), 23.0 (Leu CH'_3), 24.8 (γ-C), 28.4 (Boc CH_3), 30.2 (C-3), 35.8 (C-2), 41.4 (β-C), 54.0 (α-C), 80.5 (Boc *tert*-C), 120.4 (Ar C-3 and C-5), 128.9 (Ar C-2 and C-6), 136.4 (Ar C-1), 156.9 (C=O, carbamate), 176.5 (C=O, amide), 178.2 (C=O, acid); MS (FAB+) *m/z* 379 ([M+H]$^+$, 33%), 323 (100%).

8-*O*-(3-[4-{N_α-*tert*-Butoxycarbonyl-L-leucinoylamino}-phenyl]propanoyl)-8-*O*-debutanoylthapsigargin (11). To a mixture of **10** (0.26 mmol), **2** (0.26 mmol), and DMAP (0.03 mmol) in dry CH_2Cl_2 (2.0 mL) was added a solution of DCC (0.29 mmol) in dry CH_2Cl_2 (0.75 mL) at 0 °C. The mixture was left for 1 h at 0 °C and then 7.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent F) yielded **11** (50%) as a white amorphous solid: 3-(4-[N_α-*tert*-butoxycarbonyl-L-leucinoylamino]phenyl)propanoyl ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.3 Hz, 3H, Leu CH_3), 0.97 (d, J = 6.3 Hz, 3H, Leu CH'_3) 1.40 (s, 9H, Boc CH_3), 1.62 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.56 (t, J = 7.0 Hz, 2H, H-2), 2.83 (t, J = 7.0 Hz, 2H, H-3), 4.34 (m, 1H, α-H), 7.00 (d, J = 8.0 Hz, 2H, Ar H-2 and H-2′), 7.35 (d, J = 8.0 Hz, 2H, Ar H-3 and H-3′); ^{13}C NMR ($CDCl_3$) δ 23.0 (Leu CH'_3 and CH_3), 24.8 (γ-C), 28.4 (Boc CH_3), 29.8 (C-3), 36.2 (C-2), 40.9 (β-C), 53.9 (α-C), 80.6 (Boc *tert*-C), 120.5 (Ar C-3 and C-5), 128.7 (Ar C-2 and C-6), 136.3 (Ar C-4), 156.7 (C=O, carbamate), 172.0 (C=O, C-1), 176.5 (C=O, amide); HRMS (FAB+) *m/z* 963.4902 ([M+Na]$^+$, $C_{50}H_{73}N_2O_{15}Na$ requires 963.4830).

8-*O*-(3-[4-{L-Leucinoylamino}phenyl]propanoyl)-8-*O*-debutanoylthapsigargin (5). TFA (1.00 mL) was added to a solution of **11** (0.12 mmol) in dry CH_2Cl_2 (2.0 mL) at room temperature. The mixture was stirred for 1 h at room temperature and concentrated in vacuo to give **5** (yield 100%) as a yellowish amorphous solid: 3-(4-[L-leucinoylamino]phenyl)-propanoyl ^{1}H NMR ($CDCl_3$) δ 0.86 (m, 6H, Leu CH_3 and CH'_3), 1.59 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.58 (br s, 2H, H-2), 2.83 (br s, 2H, H-3), 4.21 (m, 1H, α-H), 7.02 (br s, 2H, Ar H-2 and H-6), 7.22 (br s, 2H, Ar H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ 23.0 (Leu CH'_3 and CH_3), 24.8 (γ-C), 29.8 (C-3), 36.2 (C-2), 40.9 (β-C), 53.9 (α-C), 120.5 (Ar C-3 and C-5), 128.7 (Ar C-2 and C-6), 136.3 (Ar C-4), 172.0 (C=O, C-1), 176.5 (C=O, amide); HRMS (FAB+) *m/z* 841.4551 ([M+H]$^+$, $C_{45}H_{65}N_2O_{13}$ requires 841.4487).

6-*tert*-Butoxycarbonylaminohexanoic Acid (12f). A solution of sodium hydroxide (1.47 mmol) in water (0.3 mL) was added to a solution of 6-aminohexanoic acid (1.50 mmol) in *tert*-butyl alcohol (3.0 mL), and the solution was left for 10 min at room temperature. Di-*tert*-butyl-dicarbonate (1.65 mmol) dissolved in *tert*-butyl alcohol (2.8 mL) was added to the solution, and the mixture was left overnight at room temperature. The mixture was concentrated in vacuo, and the residue was suspended in water (2.4 mL). The suspension was cooled on ice and acidified (pH 2) with 2 M H_2SO_4. The aqueous suspension was quickly extracted three times with EtOAc (3.6 mL), and the combined organic phases were washed three times with water (2.4 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification of the residue by NPCC (eluent B) afforded **12f** (yield 61%) as white crystals: mp 38−39 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 2H, H-4), 1.40 (s, 9H, Boc CH_3), 1.50 (m, 2H, H-3 or H-5), 1.57 (m, 2H, H-3 or H-5), 2.28 (t, J = 7.4 Hz, 2H, H-2), 3.01 (m, 2H, H-6).

Compounds **12g−j** were prepared as described for **12f** using 7-aminoheptanoic acid, 8-aminooctanoic acid, 11-aminoundecanoic acid, and 12-aminododecanoic acid, respectively, as starting materials.

7-*tert*-Butoxycarbonylaminoheptanoic Acid (12g). NPCC (eluent A) afforded **12g** (yield 82%) as white crystals: mp 55−56 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (m, 4H, H-4 and H-5), 1.45 (s, 9H, Boc CH_3), 1.48 (m, 2H, H-3 or H-6), 1.64 (m, 2H, H-3 or H-6), 2.35 (t, J = 7.5 Hz, 2H, H-2), 3.10 (m, 2H, H-7).

8-*tert*-Butoxycarbonylaminooctanoic Acid (12h). NPCC (eluent A) afforded **12h** (yield 79%) as white crystals: mp 56−57 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H, H-4 to H-6), 1.45 (s, 9H, Boc CH_3), 1.46 (m, 2H, H-3 or H-7), 1.63 (m, 2H, H-3 or H-7), 2.34 (t, J = 7.4 Hz, 2H, H-2), 3.10 (m, 2H, H-8).

11-*tert*-Butoxycarbonylaminoundecanoic Acid (12i). NPCC (eluent A) afforded **12i** (yield 54%) as white crystals: mp 67−68 °C; ^{1}H NMR ($CDCl_3$) δ 1.28 (br s, 12H, H-4 to H-9), 1.45 (br s, 9H, Boc CH_3), 1.53 (m, 2H, H-3 or H-10), 1.63 (m, 2H, H-3 or H-10), 2.34 (t, J = 7.5 Hz, 2H, H-2), 3.10 (m, 2H, H-11).

12-*tert*-Butoxycarbonylaminododecanoic Acid (12j). NPCC (eluent B) afforded **12j** (yield 33%) as white crystals: mp 83.5−84.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.27 (br s, 14H, H-4 to H-10), 1.45 (br s, 9H, Boc CH_3), 1.50 (m, 2H, H-3 or H-11), 1.63 (m, 2H, H-3 or H-11), 2.35 (t, J = 7.4 Hz, 2H, H-2), 3.10 (m, 2H, H-12).

8-*O*-(6-*tert*-Butoxycarbonylaminohexanoyl)-8-*O*-debutanoylthapsigargin (13f). Compound **2** (0.18 mmol), **12f** (0.20 mmol), and DMAP (0.20 mmol) was dissolved in dry CH_2Cl_2 (1.5 mL) at room temperature. After cooling on ice, a solution of DCC (0.20 mmol) in dry CH_2Cl_2 (0.5 mL) was added. The mixture was kept on ice for 1 h and then left for 5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent E) afforded **13f** (yield 54%) as a white amorphous solid: 6-*tert*-Butoxycarbonylaminohexanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 2H, H-4), 1.43 (s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-5), 2.32 (m, 2H, H-2), 3.09 (m, 2H, H-6); ^{13}C NMR ($CDCl_3$) δ 25.2 (C-3), 25.8 (C-4), 28.7 (Boc CH_3), 29.5 (C-5), 34.8 (C-2), 40.7 (C-6), 80.1 (Boc *tert*-C), 172.7 (C=O, C-1); HRMS (FAB+) *m/z* 816.4139 ([M+Na]$^+$, $C_{41}H_{63}NO_{14}Na$ requires 816.4146).

Compounds **13g−j** were prepared as described for **13f**, using compounds **12g−j**, respectively, as starting materials.

8-*O*-(7-*tert*-Butoxycarbonylaminoheptanoyl)-8-*O*-debutanoylthapsigargin (13g). RPCC (eluent F) afforded **13g** (yield 41%) as a white amorphous solid: 7-*tert*-Butoxycarbonylaminoheptanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 4H, H-4 to H-5), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-6), 2.29 (m, 2H, H-2), 3.07 (m, 2H, H-7); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 28.6 (Boc CH_3), 29.0 (C-4 and C-5), 32.0 (C-6), 34.4 (C-2), 40.0 (C-7), 80.0 (Boc *tert*-C), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 830.4419 ([M+Na]$^+$, $C_{42}H_{65}NO_{14}Na$ requires 830.4303).

8-*O*-(8-*tert*-Butoxycarbonylaminooctanoyl)-8-*O*-debutanoylthapsigargin (13h). RPCC (eluent F) afforded **13h** (yield 52%) as a white amorphous solid: 8-*tert*-Butoxycarbonylaminooctanoyl ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H, H-4 to H-6), 1.43 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-7), 2.29 (m, 2H, H-2), 3.07 (m, 2H, H-8); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 28.4 (Boc CH_3), 28.9 (C-4 to C-6), 31.6 (C-7), 34.3 (C-2), 40.3 (C-8), 79.8 (Boc *tert*-C), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 844.4340 ([M+Na]$^+$, $C_{43}H_{67}NO_{14}Na$ requires 844.4459).

8-*O*-(11-*tert*-Butoxycarbonylaminoundecanoyl)-8-*O*-debutanoylthapsigargin (13i). RPCC (eluent F) afforded **13i** (yield 64%) as a white amorphous solid: 11-*tert*-Butoxycarbonylaminoundecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 12H, H-4 to H-9), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-10), 2.29 (m, 2H, H-2), 3.08 (m, 2H, H-11); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 28.6 (Boc CH_3), 29.1 (C-4 to C-8), 31.8 (C-10), 34.8 (C-2), 172.8 (C=O, C-1); HRMS (FAB+) *m/z* 886.5028 ([M+Na]$^+$, $C_{46}H_{73}NO_{14}Na$ requires 886.4929).

8-*O*-(12-*tert*-Butoxycarbonylaminododecanoyl)-8-*O*-debutanoylthapsigargin (13j). RPCC (eluent I) afforded **13j** (yield 77%) as a white amorphous solid: 12-*tert*-Butoxycarbonylaminododecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (m, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-11), 2.28 (m, 2H, H-2), 3.09 (m, 2H, H-12); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 28.5 (Boc CH_3), 29.1 (C-4 to C-9), 31.7 (C-11), 34.6 (C-2), 41.0 (C-12), 172.8 (C=O, C-1); HRMS (FAB+) *m/z* 900.5084 ([M+Na]$^+$, $C_{47}H_{75}NO_{14}Na$ requires 900.5085).

8-*O*-(6-Aminohexanoyl)-8-*O*-debutanoylthapsigargin (6f). TFA (0.5 mL) was added to a solution of **13f** (0.05 mmol) in dry CH_2Cl_2 (3.0 mL) at room temperature. The mixture was left for 45 min at room temperature. Evaporation in vacuo afforded **6f** (yield 100%) as an amorphous yellowish solid: 6-Aminohexanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 2H, H-4), 1.60 (m, 4H, H-3 and H-5), 2.31 (m, 2H, H-2), 2.97 (m, 2H, H-6); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 31.8 (C-5), 34.3 (C-2), 173.0 (C=O, C-1); HRMS (FAB+) *m/z* 694.3809 ([M+H]$^+$, $C_{36}H_{56}NO_{12}$ requires 694.3802).

Compounds **6g–j** were prepared as described for **6f**, using compounds **13g–j**, respectively, as starting materials.

8-*O*-(7-Aminoheptanoyl)-8-*O*-debutanoylthapsigargin (6g). Amorphous yelowish solid (yield 100%): 7-Aminoheptanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 4H, H-4 to H-5), 1.60 (m, 4H, H-3 and H-6), 2.30 (m, 2H, H-2), 2.99 (m, 2H, H-7); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 29.0 (C-4 to C-5), 31.9 (C-6), 34.4 (C-2), 40.3 (C-7), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 708.3965 ([M+H]$^+$, $C_{37}H_{58}NO_{12}$ requires 708.3959).

8-*O*-(8-Aminooctanoyl)-8-*O*-debutanoylthapsigargin (6h). Amorphous yellowish solid (yield 100%): 8-Aminooctanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 6H, H-4 to H-6), 1.60 (m, 4H, H-3 and H-7), 2.28 (m, 2H, H-2), 3.00 (m, 2H, H-8); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 29.0 (C-4 to C-6), 31.6 (C-7), 34.8 (C-2), 40.3 (C-8), 173.1 (C=O, C-1); HRMS (FAB+) *m/z* 722.4113 ([M+H]$^+$, $C_{38}H_{60}NO_{12}$ requires 722.4116).

8-*O*-(11-Aminoundecanoyl)-8-*O*-debutanoylthapsigargin (6i). Amorphous yellowish solid (yield 100%): 11-Aminoundecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (m, 12H, H-4 to H-9), 1.59 (m, 4H, H-3 and H-10), 2.29 (m, 2H, H-2), 2.97 (m, 2H, H-11); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 28.8 (C-4 to C-9), 31.6 (C-10), 34.4 (C-2), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 764.4655 ([M+H]$^+$, $C_{41}H_{66}NO_{12}$ requires 764.4585).

8-*O*-(12-Aminododecanoyl)-8-*O*-debutanoylthapsigargin (6j). Amorphous yellowish solid (yield 100%): 12-Aminododecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 14H, H-4 to H-10), 1.60 (m, 4H, H-3 and H-11), 2.30 (m, 2H, H-2), 3.00 (m, 2H, H-12); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 29.1 (C-4 to C-10), 31.7 (C-11), 34.3 (C-2), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 778.4700 ([M+H]$^+$, $C_{42}H_{68}NO_{12}$ requires 778.4742).

6-Aminohexanoic Acid Methyl Ester Hydrochloride (14f). Thionyl chloride (4.0 mL) was slowly added to dry MeOH (30 mL) at −10 °C. After 10 min at −10 °C to the solution was added 6-aminohexanoic acid (15.25 mmol), and the mixture was left overnight at room temperature. The solution was concentrated in vacuo, and the residue was dissolved in MeOH (15 mL). To the solution was added Et_2O (60 mL) to precipitate the methyl ester hydrochloride. Filtration afforded **14f** (yield 84%) as white crystals: mp 118−122 °C; ^{1}H NMR (CD_3OD) δ 1.44 (m, 2H, H-4), 1.69 (m, 4H, H-3, and H-5), 2.37 (t, J = 7.5 Hz, 2H, H-2), 2.93 (t, J = 7.5 Hz, 2H, H-6), 3.66 (s, 3H, OCH_3); ^{13}C NMR (CD_3OD) δ 25.4 (C-3), 26.9 (C-4), 28.3 (C-2), 34.5 (C-5), 40.7 (C-6), 52.2 (OCH_3), 175.9 (C=O, C-1).

12-Aminododecanoic Acid Methyl Ester Hydrochloride (14j). Thionyl chloride (4.0 mL) was slowly added to dry MeOH (75 mL) at −10 °C. After 10 min at −10 °C, to the solution was added 12-aminododecanoic acid (13.93 mmol), and the mixture was left overnight at room temperature. The solution was concentrated in vacuo, and the residue was dissolved in MeOH (50 mL). To the solution was added Et_2O (80 mL) to precipitate the methyl ester hydrochloride. Filtration afforded **14j** (yield 93%) as white crystals: mp 160−161 °C; ^{1}H NMR (CD_3OD) δ 1.34 (m, 14H, H-4 to H-10), 1.62 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 2.91 (t, J = 7.5 Hz, 2H, H-12), 3.65 (s, 3H, OCH_3); ^{13}C NMR (CD_3OD) δ 26.1 (C-3), 27.5 (C-10), 28.6, 30.2, 30.3, 30.4, 30.5, 30.6, 30.6 (C-2 and C-4 to C-9), 34.9 (C-11), 40.9 (C-12), 52.1 (OCH_3), 176.3 (C=O, C-1).

6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoic Acid Methyl Ester (15k). N_α-*tert*-Butoxycarbonyl-L-leucine (5.50 mmol), **14f** (5.50 mmol), and DIPEA (5.50 mmol) was dissolved in dry CH_2Cl_2 (16.5 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (6.00 mmol) in dry CH_2Cl_2 (6.0 mL). After 3 h at room temperature, the mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by NPCC (eluent C) afforded **15k** (yield 50%) as a yellowish oil: ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.5 Hz, 3H, Leu CH_3), 0.94 (d, J = 6.5 Hz, 3H, Leu CH'_3), 1.34 (m, 2H, H-4), 1.44 (s, 9H, Boc CH_3), 1.51 (m, 2H, H-5), 1.64 (m, 5H, H-3, β-H and γ-H), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.24 (m, 2H, H-6), 3.67 (s, 3H, OCH_3), 4.09 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 23.0 (Leu CH'_3), 24.5 (γ-C), 24.8 (C-3), 26.3 (C-4), 28.4 (Boc CH_3), 29.2 (C-5), 33.9 (C-2), 39.2 (β-C), 41.4 (C-6), 51.6 (OCH_3), 53.2 (α-C), 80.0 (Boc *tert*-C), 156.1 (C=O, carbamate), 173.0 (C=O, C-1), 174.3 (C=O, amide); HRMS (FAB+) *m/z* 359.2522 ([M+H]$^+$, $C_{18}H_{35}N_2O_5$ requires 359.2546).

Compounds **15l,m** were prepared as described for **15k**, using N_α-*tert*-butoxycarbonyl-L-leucine and N_α-*tert*-butoxycarbonyl-L-alanine, respectively, together with compound **14j** as starting materials.

12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoic Acid Methyl Ester (15l). NPCC (eluent C) afforded **15l** (yield 53%) as white crystals: mp 63−64 °C; ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH'_3), 1.26 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.48 (m, 3H, β-H and γ-H), 1.63 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.23 (m, 2H, H-12), 3.67 (s, 3H, OCH_3), 4.08 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.2 (Leu CH_3), 22.9 (Leu CH'_3), 24.8 (γ-C), 25.0 (C-3), 26.9 (C-10), 28.4 (Boc CH_3), 29.2, 29.3, 29.5 (C-4 to C-9 and C-11), 34.2 (C-2), 39.5 (β-C), 41.4 (C-12), 51.6 (OCH_3), 53.2 (α-C), 80.1 (Boc *tert*-C), 156.1 (C=O, carbamate), 172.8 (C=O, C-1), 174.7 (C=O, amide); HRMS (FAB+) *m/z* 443.3517 ([M+H]$^+$, $C_{24}H_{47}N_2O_5$ requires 443.3485).

12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoic Acid Methyl Ester (15m). NPCC (eluent C) afforded **15m** (yield 55%) as white crystals: ^{1}H NMR (CD_3OD) δ 1.30 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.49 (m, 3H, Ala CH_3), 1.59 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.17 (m, 2H, H-12), 3.65 (s, 3H, OCH_3), 3.99 (m, 1H, α-H); ^{13}C NMR (CD_3OD) δ 18.6 (β-C), 26.1 (C-3), 28.0 (C-10), 28.8 (Boc CH_3), 30.3, 30.5, 30.7 (C-4 to C-9 and C-11), 34.9 (C-2), 40.4 (C-12), 51.8 (OCH_3), 52.1 (α-C), 80.7 (Boc *tert*-C), 158.2 (C=O, carbamate), 176.3 (C=O, amide); HRMS (FAB+) *m/z* 401.3036 ([M+H]$^+$, $C_{21}H_{41}N_2O_5$ requires 401.3015).

6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoic Acid (16k). 2 M NaOH (10 mL) was added to a solution of **15k** (0.5 mmol) in MeOH (20 mL), and the mixture was left for 40 min at room temperature. The MeOH was removed in vacuo, and the aqueous residue was cooled on ice and acidified to pH 2 with 2 M H_2SO_4. The aqueous solution was extracted

three times with EtOAc (50 mL) and the combined organic phases were washed with 10% w/v NaCl (25 mL) and water (25 mL). The organic phase was dried ($MgSO_4$) and filtered. Concentration in vacuo afforded **16k** (yield 90%) as white crystals: mp 100.5−102.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.91 (d, J = 4.5 Hz, 3H, Leu CH_3), 0.93 (d, J = 4.5 Hz, 3H, Leu CH′$_3$), 1.37 (m, 2H, H-4), 1.43 (s, 9H, Boc CH_3), 1.51 (m, 3H, β-H and γ-H), 1.65 (m, 4H, H-3 and H-5), 2.34 (t, J = 7.5 Hz, 2H, H-2), 3.24 (m, 2H, H-6), 4.15 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 22.8 (Leu CH′$_3$), 24.4 (γ-C), 24.8 (C-3), 26.2 (C-4), 28.4 (Boc CH_3), 29.0 (C-5), 33.9 (C-2), 39.3 (β-C), 41.3 (C-6), 53.2 (α-C), 80.3 (Boc *tert*-C), 156.4 (C=O, carbamate), 173.3 (C=O, amide), 177.9 (C=O, C-1); HRMS (FAB+) *m/z* 345.2430 ([M+H]$^+$, $C_{17}H_{33}N_2O_5$ requires 345.2389).

Compounds **16l,m** were prepared as described for **16k**, using compounds **15l,m**, respectively, as starting materials.

12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoic Acid (16l). Yellowish oil (yield 95%): ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH′$_3$), 1.27 (br s, 14H, H-4 to H-10), 1.43 (s, 9H, Boc CH_3), 1.48 (m, 3H, β-H and γ-H), 1.62 (m, 4H, H-3 and H-11), 2.35 (t, J = 7.5 Hz, 2H, H-2), 3.23 (m, 2H, H-12), 4.13 (br s, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 22.8 (Leu CH′$_3$), 24.7 (γ-C), 24.8 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.7, 28.9, 29.1, 29.3 (C-4 to C-9 and C-11), 34.0 (C-2), 39.5 (β-C), 41.3 (C-12), 53.1 (α-C), 80.3 (Boc *tert*-C), 156.4 (C=O, carbamate), 173.1 (C=O, amide), 178.3 (C=O, C-1); HRMS (FAB+) *m/z* 429.3356 ([M+H]$^+$, $C_{23}H_{45}N_2O_5$ requires 429.3328).

12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoic Acid (16m). Amorphous solid (yield 93%): ^{1}H NMR (CD_3OD) δ 1.30 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.49 (m, 3H, Ala CH_3), 1.59 (m, 4H, H-3 and H-11), 2.27 (t, J = 7.5 Hz, 2H, H-2), 3.18 (m, 2H, H-12), 4.00 (m, 1H, α-H); ^{13}C NMR (CD_3OD) δ 18.8 (β-C), 26.3 (C-3), 28.2 (C-10), 28.9 (Boc CH_3), 30.7, 30.9, 31.1 (C-4 to C-9 and C-11), 35.2 (C-2), 40.6 (C-12), 52.0 (α-C), 80.8 (Boc *tert*-C), 175.8 (C=O, amide), 177.8 (C=O, C-1); HRMS (FAB+) *m/z* 387.2807 ([M+H]$^+$, $C_{20}H_{39}N_2O_5$ requires 387.2859).

8-*O*-(6-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]hexanoyl)-8-*O*-debutanoylthapsigargin (17k). Compound **2** (0.36 mmol), **16k** (0.36 mmol), and DMAP (0.04 mmol) was dissolved in dry CH_2Cl_2 (2.0 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (0.40 mmol) in dry CH_2Cl_2 (1.0 mL). The mixture was left on ice for 1 h and then left for 3.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent E) afforded **17k** (yield 69%) as a white amorphous solid: 6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoyl ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H, Leu CH_3 and CH′$_3$), 1.28 (br s, 2H, H-4), 1.42 (br s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-5), 2.30 (m, 2H, H-2), 3.20 (m, 2H, H-6), 4.06 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH′$_3$), 24.8 (γ-C), 24.9 (C-3), 28.4 (Boc CH_3), 29.1 (C-4), 31.7 (C-5), 34.3 (C-2), 38.4 (β-C), 41.2 (C-6), 53.1 (α-C), 80.1 (Boc *tert*-C) 156.3 (C=O, carbamate), 172.9 (C=O, C-1), 173.6 (C=O, amide); HRMS (FAB+) *m/z* 907.5177 ([M+H]$^+$, $C_{47}H_{75}N_2O_{15}$ requires 907.5167).

Compounds **17l,m** were prepared as described for **17k**, using compounds **16l,m**, respectively, as starting materials.

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (17l). RPCC (eluent D) afforded **17l** (yield 94%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H, Leu CH_3 and CH′$_3$), 1.26 (br s, 14H, H-4 to H-10), 1.42 (br s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-11), 2.28 (m, 3H, H-2), 3.20 (m, 2H, H-12), 4.05 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH′$_3$), 24.8 (γ-C), 24.9 (C-3), 26.7 (C-10), 28.4 (Boc CH_3), 29.0−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 53.1 (α-C), 80.1 (Boc *tert*-C), 156.2 (C=O, carbamate), 172.9 (C=O, C-1), 173.0 (C=O, amide); HRMS (FAB+) *m/z* 1013.5938 ([M+Na]$^+$, $C_{53}H_{86}N_2O_{15}Na$ requires 1013.5926).

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-alaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (17m). RPCC (eluent F) afforded **17m** (yield 78%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.43 (s, 9H, Boc CH_3), 1.47 (m, 3H, Ala CH_3), 1.60 (m, 4H, H-3 and H-11), 2.28 (m, 2H, H-2), 3.22 (m, 2H, H-12), 4.12 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 18.2 (β-C), 24.9 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.8, 29.1, 29.5 (C-4 to C-9), 31.7 (C-11), 34.5 (C-2), 50.0 (α-C), 80.6 (Boc *tert*-C), 173.0 (C=O, C-1 and amide); HRMS (FAB+) *m/z* 949.5622 ([M+H]$^+$, $C_{50}H_{81}N_2O_{15}$ requires 949.5637).

8-*O*-(6-[L-Leucinoylamino]hexanoyl)-8-*O*-debutanoylthapsigargin (17k). TFA (1.2 mL) was added to a solution of **17k** (0.20 mmol) in dry CH_2Cl_2 (3.0 mL) at room temperature. The mixture was left for 45 min at room temperature. Evaporation in vacuo afforded **7k** (yield 100%) as an amorphous yellowish solid: 6-(L-Leucinoylamino)hexanoyl ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH′$_3$), 1.28 (m, 2H, H-4), 1.60 (m, 4H, H-3 and H-5), 2.29 (m, 2H, H-2), 3.20 (m, 2H, H-6), 3.62 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH′$_3$), 24.8 (γ-C), 24.9 (C-3), 29.0 (C-4), 31.7 (C-5), 34.3 (C-2), 38.3 (β-C), 44.3 (C-6), 53.6 (α-C), 170.8 (C=O, C-1), 172.9 (C=O, amide); HRMS (FAB+) *m/z* 807.4624 ([M+H]$^+$, $C_{42}H_{67}N_2O_{13}$ requires 807.4643).

Compounds **7l,m** were prepared as described for **7k**, using compounds **17l,m**, respectively, as starting materials.

8-*O*-(12-[L-Leucinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7l). Amorphous yellowish solid (yield 100%): 12-(L-Leucinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 0.95 (m, 6H, Leu CH_3 and CH′$_3$), 1.25 (br s, 14H, H-4 to H-10), 1.61 (m, 4H, H-3 and H-11), 2.33 (m, 2H, H-2), 3.25 (m, 2H, H-12), 4.19 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH′$_3$), 24.6 (C-3), 24.8 (γ-C), 26.5 (C-10), 28.8−29.1 (C-4 to C-9), 31.7 (C-11), 34.4 (C-2), 38.1 (β-C), 40.5 (C-12), 53.3 (α-C), 173.1 (C=O, C-1), 174.5 (C=O, amide); HRMS (FAB+) *m/z* 891.5641 ([M+H]$^+$, $C_{48}H_{79}N_2O_{13}$ requires 891.5582).

8-*O*-(12-[L-Alaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7m). Amorphous yellowish solid (yield 100%): 12-(L-Alaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.24 (m, 14H, H-4 to H-10), 1.53 (m, 3H, Ala CH_3), 1.57 (m, 4H, H-3 and H-11), 2.30 (m, 2H, H-2), 3.20 (br s, 2H, H-12), 4.22 (br s, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 17.4 (β-C), 24.9 (C-3), 26.8 (C-10), 28.8, 29.3, 29.6 (C-4 to C-9), 31.8 (C-11), 34.6 (C-2), 50.4 (α-C), 174.1 (C=O, amide); HRMS (FAB+) *m/z* 849.5057 ([M+H]$^+$, $C_{45}H_{73}N_2O_{13}$ requires 849.5112).

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-serinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (18n). *N*-*tert*-Butoxycarbonyl-L-serine (0.18 mmol), **6j** (0.18 mmol), and HOBT (0.18 mmol) were dissolved in dry DMF (2.0 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (0.18 mmol) in dry DMF (1.0 mL). The mixture was left on ice for 1 h and then left for 3.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent J) afforded **18n** (yield 72%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-serinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (br s, 14H, H-4 to H-10), 1.45 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-11), 2.29 (m, 2H, H-2), 3.24 (t, J = 6.2 Hz, 2H, H-12), 3.64 (m, 1H, β-H′), 4.05 (dd, J = 3.0 and 11.1 Hz, 1H, β-H), 4.11 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) 24.9 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.8−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 39.6 (C-12), 62.8 (β-C), 80.8 (Boc *tert*-C), 156.6 (C=O, carbamate), 171.6 (C=O, C-1), 173.2 (C=O, amide); HRMS (FAB+) *m/z* 965.5593 ([M+H]$^+$, $C_{50}H_{81}N_2O_{16}$ requires 965.5586).

Compound **18o** was prepared as described for **18n**, using N_α-*tert*-butoxycarbonyl-L-phenylalanine as starting material.

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-phenylalaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (18o). RPCC (eluent J) afforded **18o** (yield 73%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-phenylalaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.38 (br s, 9H, Boc CH_3), 1.58 (m, 6H, H-3 and H-11), 2.28 (m, 3H, H-2), 3.01 (m, 2H, β-H), 3.13 (m, 2H, H-12), 4.25 (m, 1H, α-H), 7.18−7.29 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 28.3 (Boc CH_3), 29.0−29.3 (C-4 to C-9), 31.7

(C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 62.0 (α-C), 81.4 (Boc *tert*-C), 127.0 (Phe C-4), 128.8 (Phe C-2, C-2′), 129.5 (Phe C-3, C-3′), 137.0 (Phe C-1), 158.3 (C=O, carbamate), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 1025.606 ([M+H]$^+$, $C_{56}H_{85}N_2O_{15}$ requires 1025.595).

Compounds **7n,o** were prepared as described for **7k**, using compounds **18n,o**, respectively, as starting materials.

8-*O*-(12-[L-Serinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7n). Amorphous yellowish solid (yield 100%): 12-(L-Serinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.58 (m, 4H, H-3 and H-11), 2.29 (m, 2H, H-2), 3.17 (br s, 2H, H-12), 3.74 (dd, J = 13.7 and 6.8 Hz, 1H, β-H′), 3.88 (br s, 1H, α-H), 4.01 (br s, 1H, β-H); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.6 (C-10), 28.8−29.1 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 55.4 (α-C), 59.6 (β-C), 173.0 (C=O, C-1), 174.4 (C=O, amide); HRMS (FAB+) *m/z* 865.5010 ([M+H]$^+$, $C_{45}H_{73}N_2O_{14}$ requires 865.5062).

8-*O*-(12-[L-Phenylalaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7o). Amorphous yellowish solid (yield 100%): 12-(L-Phenylalaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 14H, H-4 to H-10), 1.60 (m, 6H, H-3 and H-11), 2.28 (m, 2H, H-2), 2.68 (dd, J = 9.3 and 13.7 Hz, 1H, β-H), 3.23 (m, 3H, β-H′ and H-12), 3.58 (dd, J = 9.3 and 4.2 Hz, 1H, α-H), 7.20−7.34 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 29.0−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 61.1 (α-C), 127.0 (Phe C-4), 128.9 (Phe C-2, C-2′), 129.5 (Phe C-3, C-3′), 138.1 (Phe C-1), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 925.539 ([M+H]$^+$, $C_{51}H_{77}N_2O_{13}$ requires 925.543).

Isolation of Sarcoplasmic Reticulum (SR). Frozen rabbit muscle was purchased from Pel-Freez Biologicals (Rogers, AR) and MOPS, sucrose, EDTA, and KCl were purchased from SIGMA. Homogenization was done with a commercial blender (Waring, USA). Centrifugation was done with a Sorvall RC-5B superspeed centrifuge (DuPont, USA) and a L7 ultracentrifuge (Beckman Coulter, USA). The temperature was kept at 0−4 °C during the preparation. Frozen rabbit muscle (180 g) was blended 15 s every 5 min during 1 h with 510 mL of a solution containing 10 mM MOPS, pH 7.0, 10% sucrose and 0.1 mM EDTA. The pH was kept between 6.5 and 7.0 by adding 10% NaOH. The homogenate was centrifuged at 15000*g* for 20 min. The supernatant was filtered through a path of cheesecloth, and centrifuged at 40000*g* for 90 min. The pellet was suspended with a Dounce glass homogenizer in 60 mL of a solution containing 10 mM MOPS, pH 7.0, and 0.6 M KCl. After incubating for 40 min at 4 °C, the suspension was centrifuged at 15000*g* for 20 min. The 10% top of the supernatant and the pellet were discarded. The supernatant was collected and centrifuged at 40000*g* for 90 min. The pellet was suspended with a Dounce glass homogenizer in 40 mL of microsome storage solution containing 10 mM MOPS, pH 7.0 and 30% sucrose. The microsomes were stored at −80 °C. The sarcoplasmic reticulum (SR) protein concentration (1.1 mg/mL) was determined with the Micro BCA Protein Assay Reagent kit supplied by Pierce (Rockford, IL) using bovine serum albumin (BSA) as standard.

Measurement of ATPase Activity. KCl, Trizma-HCl, $MgCl_2$, EGTA, $CaCl_2$, β-NADH, phosphoenolpyruvate (PEP), A23187, phosphoenolpyruvate kinase (PK), lactate dehydrogenase (LDH), and ATP were supplied by Sigma. The ATPase activity was measured with a Spectramax Plus^{384} microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA) as the rate of ATP hydrolysis essentially as previously described.[66,128] Buffer A: 0.1 M KCl, 20 mM Trizma-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.7 mM $CaCl_2$. Solution 1: 1.2 mM β-NADH, 1.5 mM PEP, 4.5 μM A23187, 22.5 U/mL PK, 54 U/mL LDH and 30 μg/mL SR protein in buffer A. Solution 2: Control or inhibitor dilutions in buffer A (concentrations corrected for a 1:3 dilution). Solution 3: 0.72 mM ATP in buffer A. 100 μL of solution 1 was mixed with 100 μL of solution 2 and 100 μL of solution 3 was added to start the reaction. After 5 min of incubation, the OD_{340} was measured kinetically at room temperature (n = 3) for at least 10 min. Typically, a 1 mM DMSO solution of inhibitor was diluted 1:100 in buffer A before making serial dilutions in buffer A. The amount of DMSO present did not influence the measured ATPase activity. The total ATPase activity was 7.0 μmol of ATP (mg of SR protein)$^{-1}$ min^{-1}.

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Appendix 2
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Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer

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See "Notes" following "References."

Abstract

Background: **Androgen independent prostate cancer cells within metastatic sites have a remarkable low rate of cell proliferation consistent with their relative unresponsiveness to standard anti-proliferative chemotherapy in patients. In contrast to these standard agents, thapsigargin is a lipophilic cytotoxin that is equally effective against both highly proliferative and quiescent prostate cancer cells due to its ability to disrupt intracellular free Ca^{2+} via inhibition of a ubiquitous housekeeping protein, the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump.** ***Methods:*** **To both solubilize and target its cytotoxicity, thapsigargin has been chemically modified and coupled to a peptide carrier to produce a soluble, latent prodrug that is specifically activated extracellularly within prostate cancer sites by the unique protease, prostate-specific antigen (PSA). We have coupled a selective PSA peptide substrate to an amino acid containing thapsigargin analog termed L12ADT to produce a cell impermeant, inactive prodrug.** ***Results*****: The L12ADT peptide prodrug is efficiently hydrolyzed by PSA, stable in human plasma, and selectively toxic to PSA-producing prostate cancer cells in vitro. Pharmacokinetics revealed peak serum prodrug concentration of 15.4 ± 1.1 μM and a half-life of ~ 2.8 h. Over 24 hrs, < 0.5% of free L12ADT was observed in plasma. Within prostate cancer tissue, levels of prodrug were ~ 8-fold and liberated L12ADT were ~ 6-fold higher than the in vitro LD50's. Intravenous administration of the prodrug produces growth inhibition of PSA-producing LNCaP human prostate cancer xenografts without significant host toxicity. Continuous subcutaneous administration produces complete growth inhibition of established PSA-producing LNCaP xenografts with no effect on PSA non-producing SN12c renal carcinoma xenografts.** ***Conclusions:*** **These data validate the selectivity of targeting and warrant further development of PSA-activated TG prodrugs as therapy for metastatic prostate cancer.**

Introduction

At clinical presentation, prostate cancers are heterogeneously composed of androgen dependent and androgen independent cells (1). Following androgen ablation, the androgen dependent cells undergo apoptosis resulting in an initial beneficial clinical response (2). This is followed by relapse to a state unresponsive to further anti-androgen therapy, no matter how completely given, due to the presence of androgen independent prostate cancer cells within the metastatic sites (3). As previously demonstrated, androgen ablation does not induce apoptosis in androgen-independent prostate cancer cells due to a defect in the initiation step, even though these cells retain the basic cellular machinery to undergo apoptosis following exposure to a variety of agents (4-8).

The failure to induce apoptosis is related to the inability of androgen ablation to induce a sustained elevation in the intracellular free Ca2+ (Ca_i) levels in these androgen-independent cells. As a corollary to the inhibition studies, agents that increase Ca_i (i.e., calcium ionophores) activate apoptosis in both normal and malignant prostate cells (5-11). These observations provided the rationale for the development of targeted prodrugs that could selectively elevate Ca_i leading to induction of apoptosis of androgen-independent prostate cancer cells as new therapy for metastatic prostate cancer (5,6). Such a prodrug approach was initiated using thapsigargin (TG), [figure 1a] the active principle of the umbelliferous plant *Thapsia garganica* (12), that is a potent nM inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump (6,13,14). Inhibition of the SERCA pump by TG leads to a rapid 3-5 fold elevation in Ca_i due to emptying of the stored calcium pools within the ER due to a continuous, passive leakage of Ca^{2+} out the ER (13). Depletion of the ER Ca^{2+} pool generates a signal that induces a change in the permeability of the plasma membrane, leading to an influx of Ca^{2+} due to the high extracellular Ca^{2+} concentration (i.e. 1-3 mM) (15).

Using a method to longitudinally monitor Ca_i within individual cells for up to 7 days (16) we demonstrated that 50-100 nM TG treatment induces a biphasic elevation of Ca_i (8, 16,17). The initial elevation to 200-400 nM Ca^{2+} for 4-6 hr is followed by a return to baseline before a second elevation to micromolar levels that occurs after 24-72 hrs of TG exposure. Before this secondary Ca^{2+} rise, the expression of a series of Ca^{2+} regulated proteins are enhanced (e.g.calmodulin, PAR-4, GADD 153, GRP-78, etc.) even though general protein synthesis decreased (6, 17-19). Once this secondary rise to μmolar Ca^{2+} occurs, the calcium dependent phosphatase, calcineurin, becomes activated and dephosphorylates a series of proteins including the pro-apoptotic protein, Bad. This allows Bad to translocates from the cytoplasm to the mitochondria resulting in a Bax dependent permeability change in the mitochondrial outer membrane releasing cytochrome c and apoptosis inducing factor (AIF) (19,20). Once released, these proteins induce the activation of calpains and caspases resulting in the morphological changes and genomic DNA fragmentation associated with apoptosis. (8,16, 17,21). This second rise in Ca_i is asynchronous within the cell population but ultimately occurred in every dying cell (8,16,19).

The most important characteristic of TG is its ability to induce apoptosis in proliferatively quiescent (i.e.G_0 arrested) as well as proliferating prostate cancer cells (22). TG's cytotoxicity , however, is not prostate or cell-type specific. TG, therefore, cannot be administered systemically as a therapeutic agent without significant host toxicity. In 1993, we described a prodrug approach based on coupling a primary amine containing TG analog to a peptide carrier in order to produce a more water soluble compound that could be delivered systemically (23). This prodrug form is inactive since it cannot enter cells until the TG analog is liberated from the carrier peptide by proteolytic digestion. Both normal and malignant prostate epithelial cells secrete large amounts of the tissue restricted protease prostate-specific antigen (PSA) (24-26). PSA is a serine protease with chymotrypsin-like substrate specificity that is enzymatically active in the extracellular fluid of prostate cancers while enzymatically inactivated in the blood serum (24-26). Thus a prostate cancer targeted TG prodrug could be developed consisting of a primary amine containing analog of TG coupled via a PSA cleavable peptide bond to a PSA-specific peptide carrier (23, 27). To accomplish this, we identified a highly selective and efficient PSA substrate with the sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) (28). Using this substrate we confirmed that the extracellular fluid surrounding prostate cancers contained high concentrations of enzymatically active PSA that could hydrolyze this substrate (29). In contrast, this substrate was stable in plasma even from patients with high plasma PSA levels (29). This is because PSA in the circulation is enzymatically inactive due to complex formation with abundant serum protease inhibitors alpha-1-antichymotrypsin and alpha-2-macroglobuin (25,26, 29).

Primary amine containing TG analogs, therefore, can be coupled to the HSSKLQ peptide to form a PSA-cleavable peptide bond. We prepared TG analogs by substituting its O-8 butanoyl group with various primary amine containing side chains and characterized their ability to inhibit the SERCA pump, to elevate Ca_i in intact whole prostate cancer cells and for cytotoxicity against human prostate cancer cell lines (30,31). Initial attempts to directly couple primary amine containing toxins to the C-terminal carboxyl of glutamine produced prodrugs that were inefficiently hydrolyzed by PSA (32). Previously, however, we demonstrated that PSA could hydrolyze the toxin-peptide conjugates when the amino acid leucine was introduced as a linker and between the peptide and the primary amine containing toxin (32,33). Based on this observation, we coupled a 12-aminododecanoyl side chain analog of TG (12ADT) to leucine to produce L12ADT (figure 1a), an analog that is as potent a cytotoxin as TG (i.e. LD_{50} ~ 30 nM) (31). The goal of the

present study was to synthesize and characterize a prodrug consisting of this potent primary amine containing TG analog coupled to the PSA-specific peptide HSSKLQ, figure 1b, for kinetics of PSA hydrolysis and *in vitro* cytotoxicity as well as its *in vivo* pharmacokinetics and selectivity of its antitumor efficacy vs. host toxicity using nude mice xenograft models.

Materials and Methods:

Cell Lines. The LNCaP, DU145, and PC-3 human prostate cancer cell lines, the HCT-116 human colon cancer cell line, the TT human medullary thyroid cancer line, the MCF-7 human breast cancer line, and WI-38 normal human fibroblasts were obtained from ATCC (Rockville, MD). The C4-2B human prostate cancer cells were from UroCor, the MDA-PCA-2B human prostate cancer cells were from Dr. Nora Navone (M.D. Anderson), the LAPC-4 human prostate cancer cells were from Dr. Charles Sawyer (UCLA), the SN12C human renal cancer cells were from Dr. Isaiah Fidler (M.D. Anderson), the CWR22R human prostate cancer cells were from Dr. J. Jacobsen (Case Western Reserve) and the TSU human bladder cancer cells were from Dr. T. Itzumi (Teikyo University, Japan). The NHO normal human osteoblasts, HMVec normal human microvein endothelial cells, the HuVec normal human macrovein endothelial cells and the PREC normal human prostate epithelial cells were obtained from Clonetic (Bio Whittaker Inc, Walkersville MD)

Materials. Mu-HSSKLQ-L12ADT was produced by coupling L12ADT to the Mu-HSSK(Fmoc)LQ peptide (purchased from California Peptide Inc., Napa, CA) using HBTU in DMF. After deprotection in piperidine/DMF, prodrug was purified by HPLC then characterized by mass spectroscopy. PSA was purified from human seminal plasma as described (28). All other reagents, unless otherwise specified, were from Sigma (St. Louis, MO). Thapsigargin was ethanol extracted from the harvested seeds of *Thapsia garganica* and analogs synthesized as previously described (30). 8-O-(12[L-leucinoylamino]dodecanoyl)-8-O-debutanoyl thapsigargin (L12ADT) was synthesized from TG as described (31).

Mu-HSSKLQ-L12ADT prodrug synthesis and kinetic analysis of PSA hydrolysis: Mu-HSSK(Fmoc)LQ peptide was purchased from California Peptide, Inc. (Napa, CA). The final peptide product was purified by HPLC and analyzed by NMR and MALDI-TOF mass spectrometry. The amino terminus of the peptide was protected by coupling to a 4-morpholinecarbonyl (Mu) blocking group (F.W. 115). The lysine of the Mu-HSSKLQ peptide was protected with Fmoc group. L12ADT was coupled to peptide using HBTU in DMF. Final product was deprotected in piperidine/DMF and purified by HPLC then characterized by HPLC, NMR, and Mass Spectroscopy.

Mu-HSSKLQ-L12ADT was incubated with PSA (10 μg/ml final concentration) at various concentrations. At discrete time points (0.5, 1,2,3,4 hrs) aliquots of the reaction mixture were removed and HPLC analysis performed. Using a reversed phase C18 Ultrasphere analytical column (Beckman) 15 cm x 4.6 mm (I.D.), an isocratic method was developed to visualize the free peptide peak (9% Acetonitrile; 0.1% TFA; H_2O). A standard curve was produced using purified free Mu-HSSKLQ peptide in order to convert peak area to free peptide concentration. Peak areas of free peptide at each time point were then converted to concentration and data analyzed by Lineweaver-Burke plots [1/V (V=reaction velocity) vs. 1/S (S= substrate concentration)]. K_m, V_{max} and k_{cat} were calculated from these plots and ratio of k_{cat}/K_m was used to rank the prodrugs on the basis of efficiency of PSA hydrolysis.

Calcium Measurements: Determination of intracellular free calcium levels was performed in a cuvette assay with fura-2AM (Molecular Probes, Eugene OR) loaded TSU cells as previously described (16).

Cytochemical Staining: Intracellular caspase 3 activation and FITC-annexin V staining were performed using flow cytometry as previously described (34). The PhiPhiLux-G_1D_2 substrate was obtained from OncoImmunin (Gaithersburg, MD). Annexin V FITC apoptosis kit was obtained from BD Pharmingen (San Diego, CA). For AIF cytochemical staining, cells were cytospun onto lysine coated slides and standard DAB immunoperoxidase staining performed using polyclonal anti-AIF serum from Santa Cruz (Santa Cruz, CA).

Cytotoxicity Assays:

The cytotoxic response of LNCaP cells to 48 h exposure to 100 nM of either doxorubicin (Sigma), taxol (Sigma), or TG in standard high proliferation culture or low proliferation culture was assessed by nuclear DAPI staining as previously described (22). High versus low proliferation in these cultures was determined based on Ki-67 staining as described previously (22). Five day cytotoxic assays for cell viability were performed using the Promega Cell Titer 96 Non-Radioactive Cell Proliferation Assays, Promega Corp (Madison, WI) according to the manufacturer's instructions as we have previously described (35) to determine response to compounds at varying dose and to determine the lethal dose of compounds that produced 50% cytotoxicity (i.e. LD_{50}).

Clinical Samples. The growth fraction of human prostate cancers were determined based on Ki-67 staining of tissues from the archival collection of the Johns Hopkins School of Medicine Department of Pathology and the warm autopsy program of the NIH-SPORE program at Johns Hopkins as described previously (22, 36).

Intratumoral injection of LNCaP tumors with L12ADT. 2 x 10^6 LNCaP cells in 100 μl of Matrigel (Collaborative Research, Bethesda, MD) were inoculated into the flank of 6 week old male nude mice (Harlan). When tumors reached 0.5-1 cm^3 they were injected daily with 100 μl of either sterile saline or L12ADT. At the end of the experiment, harvested tumors were fixed, stained with hematoxylin and eosin and examined using a digital camera at 25 X magnification. The

total area and the area of viable tumor were determined using an ImagePro image analysis package as described previously (37).

Determination of plasma levels of Mu-HSSKLQ-L12ADT prodrug

Calibration standards and samples were analyzed by liquid chromatography coupled to a quadripole mass spectrometer (LC/MS/MS) [PESciex API 3000]. A multistep gradient elution HPLC method was developed to separate both the Mu-HSSKLQ-L12ADT prodrug and the L12ADT analog with eluent A= 2mM ammonium acetate with 0.1 % formic acid and eluent B= 90% acetonitrile/10% deionized water. Calibration was done using extracted standards in a range of 0.001-100 μM and linear regression analysis used to generate best fit line from which peak areas of samples were converted to concentration of prodrug or L12ADT.

Determination of tumor tissue levels of Mu-HSSKLQ-L12ADT prodrug and free L12ADT.

LNCaP bearing animals received 3 daily intravenous injections of 7mg/kg of Mu-HSSKLQ-L12ADT. At one hr after the last injection LNCaP tumor tissue were harvested and homogenized on ice in protease inhibitor containing-buffer [Complete Inhibitor (Boehringer Manheim] using a mechanical tissue grinder. A tissue calibration curve was constructed by adding an internal standard [8-*O*-(12-{L-serinoylamino}dodecanoyl)-8-*O*-debutanoylthapsigargin (S-12ADT)] (31) (i.e. final concentration range from 30 to 0.014 μM) to tumor tissue homogenate. LC-MS (Agilent) analysis was performed with mobile phase consisted of 0.1% Formic acid in an acetonitrile gradient from 5% to 100% over 16 minutes with a flow rate of 0.5mL per minute. Each compound was discriminated based on the individual extracted ion chromatogram and the areas of the MuHSSKLQ-L12ADT and L-12ADT were converted into a ratio with the internal standard.

Systemic efficacy studies: Tumor cells (LNCaP or SN12C) were inoculated into male nude mice as described above. Animals were grouped so that the average starting tumor volumes (i.e. ~ 0.1- 0.2cc) were equivalent. Tumors were measured with calipers and animals were weighed biweekly while on treatment. At the end of the experiments, animals were sacrificed by CO_2 overdose and tumor weights were obtained. For intravenous experiments, tumor bearing animals were administered a 7 mg/kg/day dose of Mu-HSSKLQ-L12ADT (2% DMSO/H2O) via daily tail vein injection. Animals were treated once a day, 5 days a week for three treatment cycles at indicated times. Controls were similarly treated with vehicle only (2% DMSO/H2O). For continuous infusion experiments, osmotic minipumps (Alzet) containing Mu-HSSKLQ-L12ADT (12.4 mg/ml in sterile H_20/10% DMSO) were inserted subcutaneously under sterile conditions through a flank incision and closed with staples. The osmotic pump delivered drug at a rate of 0.5 μl/hr for ~ 14 day for an average dose of 7 mg/kg/day. Vehicle controls were similarly treated with an osmotic pump containing only 10% DMSO/H2O. All animal studies were performed according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

Statistics: Statistical analysis of differences in growth rates in vitro and tumor volumes and weights in vivo between Mu-HSSKLQ-L12ADT and vehicle controls were performed using student t-test and p values < 0.05 reported in text.

Results

Advantage of thapsigargin in treatment of prostate cancer

A variety of agents are able to effectively induce apoptosis of human prostate cancer cells in standard in vitro assays. In such standard culture conditions the growth fraction of the human prostate cancer cell lines during drug exposure is characteristically >90% (Fig. 2a). In contrast, the growth fraction of malignant cells within metastatic tissue obtained at autopsy from patients who had failed androgen ablative therapy is <10% (Fig. 2a).

In order to more appropriately mimic the clinical situation, we have developed an assay system in which LNCaP human prostate cancer cells are shifted into a low growth fraction state by culturing in media conditioned by non-proliferating human osteoblasts (22). Using this system, the growth fraction can be shifted from ~90% to 10-20% without loss of cell viability (i.e. >95% viable cells at one week of such low growth fraction culture) (Fig 2a). This low growth fraction culture system more closely approximates cell kinetics occurring in prostate cancers in patients. The responsiveness of LNCaP cells to taxol and doxorubicin vs. TG was compared in standard high proliferation culture conditions (Fig. 2b). While all of these agents were equally effective in the high growth fraction cultures, only TG retained its effectiveness when assayed in the low proliferation cultures (Fig. 2b).

TG's cytotoxicity, however, is neither malignancy nor cell-type specific. To illustrate the generality of this potent cytotoxic response, a series of normal and malignant cells were exposed to 100 nM TG for 5 days. Such exposure resulted in an 80-90% reduction in viable cell number regardless of whether the cells are normal or malignant or of prostatic origin, (Table 1). This cytotoxic response is not limited to a particular subtype of prostate cancer and occurs regardless of the androgen receptor status or responsiveness of the cell, (Table 1). Thus, while TG could be effective therapy for prostate cancer, it cannot be administered systemically without significant host toxicity. To confirm this, mice were given increasing doses of TG intravenously. At a dose of TG of 0.8 mg/kg (i.e. 1280 nmoles/kg), all mice (n=5) died within an hour. These studies document the systemic toxicity and affirm the need for targeted delivery of TG analogs by the prodrug approach.

Targeting Thapsigargin to Prostate Cancer Cells

Since there is no inherent therapeutic index for the cytotoxic response of malignant vs. normal cell types to TG, a strategy is required for targeting TG to prostate cancer sites to prevent toxicity to normal cells. As presented in the introduction, leucine containing analogs were screened for their relative potency compared to the parent TG. L12ADT was identified as the lead compound due to its identical cytotoxic potency to TG. The cytotoxicity of L12ADT is due to its ability to rapidly enter cells and inhibit the SERCA pump producing an initial elevation of intracellular Ca^{2+}, (Fig. 3a). Like TG, L12ADT exposure results in an initial Ca_i elevation to 200-400 nM within minutes that returns to baseline (i.e. 20-40 nM) within 6-8 hr. This initial Ca_i wave is then followed by a secondary sustained elevation of > 10 μM Ca_i that occurs in cells between 18-96 hr of drug exposure (data not shown). This secondary sustained μM Ca_i elevation is associated with the translocation of cytochrome C from the cytoplasm to the mitochondria and translocation of AIF from the mitochondria to the nucleus, (Fig. 3b). These changes induce the apoptotic cascade resulting in activation of caspase 3 (Fig. 3c) and externalization of phosphotidylserine to the extracellular surface of the plasma membrane detectable using annexin V staining, (Fig. 3d). Eventually cells undergo plasma membrane blebbing and subsequent fragmentation into apoptotic bodies, (Fig. 3e,f).

To determine if L12ADT induces a similar cytotoxic effect *in vivo*, nude mice bearing LNCaP tumors received two 5 day courses separated by two off days for a total of 10 intratumoral inoculations of either vehicle or L12ADT (100 nanomoles/injection producing a theoretical intratumoral concentration of 100-200 nM) with no ill-effects upon the general health of the host mouse. The volume of viable tumor cells in the vehicle control group at the end of a 12 day period was 166 ± 5% of the starting volume, while in the L12ADT treated group the volume was 45 ± 6 % of the starting volume. These results were significant (i.e. p value <0.5) and demonstrated that L12ADT could be an effective therapy for prostate cancer without host toxicity if sufficient concentrations (i.e. 100- 200 nM) of L12ADT could be targeted to tumor sites. As expected, like TG L12ADT also possessed significant systemic toxicity [i.e. dose of 1.6 mg/kg (i.e. 2560 nmoles/kg) killed 100% mice within an hour of dosing].

In vitro characterization of L12ADT prodrugs

On the basis its systemic toxicity, L12ADT was coupled to the Mu-HSSKLQ peptide carrier to produce the prodrug Mu-HSSKLQ//L12ADT, where // denotes PSA cleavage site (Fig. 1b). Such peptide coupling prevents L12ADT from entering cells and inhibiting the SERCA pumps as demonstrated by the lack of Ca_i elevation in PSA-non-producing cells (i.e. TSU bladder cancer cells) following exposure to even 10 μM of Mu-HSSKLQ//L12ADT, figure 3a. PSA cleaved L12ADT from the prodrug peptide with a K_m of 475 μM, k_{cat} of 0.0096 s^{-1}, and k_{cat}/K_m of 21.9 $s^{-1}M^{-1}$. These kinetics were comparable to those previously reported for the fluorescent substrate Mu-HSSKLQ//AMC [i.e. K_m 470 μM, k_{cat} 0.011, k_{cat}/K_m 23.6 $s^{-1}M^{-1}$] (28). In 5 day *in vitro* exposure cytotoxicity assays, the LD_{50} of the Mu-HSSKLQ//L12ADT prodrug against PSA-producing LNCaP human prostate cancer cells was 74 ± 3 nM.

Continuous exposure of cells *in vitro* does not mimic the expected episodic exposure to prodrug following daily *in vivo* systemic administration. This is particularly relevant with regard to a cytotoxic agent like L12ADT, which, due to its high lipophilicity, is concentrated by cells from the aqueous environment and retained once liberated from the prodrug. To demonstrate this point, the response to episodically administered prodrug (i.e. 8 hr/day) was compared to the response when given continuously *in vitro,* (Fig. 4a). Continuous exposure of LNCaP cells to 500 nM prodrug resulted in nearly 2 logs of cell kill by day 7. Similar results were obtained with episodic exposure to 1000 nM, (Fig. 4a). A more delayed effect was observed following episodic exposure to 500 nM TG prodrug, (Fig. 4a). As a control for the specificity of this response, the PSA non-producing human HCT-116 colon cancer cells were exposed continuously to 500 nM prodrug. In contrast to the response of PSA-producing LNCaP cells, no significant antitumor effect was observed, even though these cells were exquisitely sensitive (i.e. ~ 2 logs of cell kill by day 5) to 500 nM of the free L12ADT, (Fig. 4b).

Pharmacokinetic Studies of the HSSKLQ//L12ADT prodrug

A sensitive LC-MS method was developed to measure levels of prodrug and L12ADT in blood of mice. The C_{max} of the prodrug at 5 minutes post single intravenous injection of 7 mg/kg was 15.4 ± 1.1 μM and the half-life was 2.8 ± 0.02 hours, figure 5. In contrast, the C_{max} for L12ADT,observed at 12 hrs post prodrug dosing was only 10.3 ± 2 nM, figure 5. The Mu-HSSKLQ//L12ADT prodrug was stable in blood with <0.5% (i.e. Ratio of AUC Prodrug to AUC L12ADT was 0.3 ± 0.03%) of the prodrug non-specifically hydrolyzed to the free L12ADT over a 24 hr period.

The data demonstrate that blood levels of the Mu-HSSKLQ//L12ADT above the in vitro LD_{50} for LNCaP cells (i.e. 75 nM) are sustained for ~ 12 hrs following a 7mg/kg intravenous injection. To determine the intratumoral levels of both prodrug and free L12ADT, animals bearing LNCaP xenografts (n=4) were injected intravenously for three consecutive days with the prodrug at 7 mg/kg and tumors harvested one hour after the third dose. The intratumoral concentration of Mu-HSSKLQ//L12ADT was 640 ± 80 nM (i.e. 8.5-fold higher than the LNCaP LD_{50} for Mu-HSSKLQ//L12ADT *in vitro*) and 170 ± 58 nM for the liberated L12ADT (i.e. 5.6-fold higher than the LNCaP LD_{50} for L12ADT *in vitro*). These results demonstrate that a dose of 7 mg/kg of prodrug can produce effective cytotoxic tumor tissue levels.

In vivo antitumor efficacy of L12ADT prodrug

On the basis of the pharmacokinetics, LNCaP bearing animals were injected intravenously with 7 mg/kg of the prodrug daily x 5 days. Control animals received injections of vehicle only. Animals received a total of three 5-day cycles of intravenous therapy on days 1-5, 14-18, and 35-39. The results demonstrate a significant inhibition of tumor growth in the prodrug treated group over the treatment period with transient tumor regression following each cycle of therapy, (Fig. 6a).

To determine if an enhanced antitumor effect could be achieved with continuous exposure to prodrug, LNCaP bearing animals received either 7 mg/kg/day of Mu-HSSKLQ//L12ADT prodrug or vehicle control for 28 days via continuous release subcutaneously implanted osmotic minipumps. The response of the two groups over a 40-day observation period demonstrate a nearly complete cessation of net tumor growth in the prodrug treated group during the period of drug exposure (1-28 days) (Fig. 6b). As an additional control, a size-matched group of animals bearing the PSA non-producing human SN12C renal cell carcinoma cell lines was treated with the same 7 mg/kg/day dose of prodrug via osmotic pump with no effect on the growth of tumors in the prodrug treated group vs. vehicle treated group (Fig. 6c). These results confirm that enzymatically active PSA must be present within the tumor for prodrug activation and subsequent antitumor effect.

In all in vivo studies there was no discernible toxicity, no significant (i.e. <15%) weight loss and no deaths in the animals treated with prodrug administered either intravenously or via osmotic minipump over the course of treatment.

Discussion:

Based on the unique biology of prostate cancer, we initially proposed that PSA could be used to target therapies selectively to metastatic prostate cancer sites within the patient (23). Such targeting required the identification of a highly selective peptide substrate for PSA (28). The Mu-HSSKLQ peptide substrate was chosen for further development based on both selective PSA hydrolysis and stability in the serum of men with high PSA levels (28). We coupled this peptide to doxorubicin to produce an inactive prodrug (32,33). Using this PSA-activated doxorubicin prodrug, we were the first group to validate that such a PSA-cleavable peptide could be used to selectively target prostate cancer cells both *in vitro* (32) and *in vivo* (33). Subsequently this concept was confirmed by Defeo-Jones at al who used a different peptide substrate with the sequence gyl-XASZQ//SL (where gyl is N-glutaryl, X is 4-hydroxylprolyl, Z is cyclohexylglycyl and // denotes site of PSA cleavage) to construct a doxorubicin prodrug (38).

In additional studies, Wong et al using this gyl-XASZQ//SL-doxorubicin prodrug demonstrated that ~ 33% of this prodrug was metabolized to free doxorubicin in the plasma of several species following intravenous administration (39). In a phase I clinical trial, similar conversion of this gyl-XASZQ//SL-doxorubicin prodrug in the blood of patients to free leucine-doxorubicin (68% conversion) and doxorubicin (19% conversion) was also observed (40). These data demonstrate that the gyl-XASZQ//SL peptide sequence is not optimal for selective targeting. In contrast, our preclinical results demonstrate that less than 1% of the Mu-HSSKLQ-L12ADT prodrug is non-specifically hydrolyzed in blood.

Even using our more selective peptide delivery, we have found that doxorubicin is not an optimal toxic agent to activate apoptosis of low proliferation prostate cancers. As demonstrated in figure 2b, neither taxol nor doxorubicin were effective in inducing apoptosis of prostate cancer cells under low proliferation conditions which better mimic the cell kinetic state characteristic of lethal prostate cancers in patients. In contrast, TG and its analogs are able to induce apoptosis regardless of the kinetic state of the target cells, figure 2b. Since, however, there is no inherent therapeutic difference between normal and malignant cells with regards to TG's induction of apoptosis, a targeted prodrug approach has been developed.

Besides its proliferation independent cytotoxicity, there are a number of additional advantages to this PSA-activated TG prodrug approach. Once liberated extracellularly, L12ADT rapidly enters cells at the site of activation due to its high degree of lipophilicity with little release into the general circulation, thus minimizing distant side effects. The PSA-activated TG prodrug approach also overcomes the problem of heterogeneity in the production of the target protease PSA by individual prostate cancer cells within a given metastatic site. Previously we demonstrated high levels (i.e. 50-500 μg/ml)of enzymatically active PSA in the extracellular fluid (ECF) of human prostate cancers (29). Therefore, a substantial cytotoxic bystander effect occurs because cleavage of the TG prodrug in the ECF of prostate cancer sites requires production of the active PSA enzyme by only a percentage of the prostate cancer cells. This extracellular activation has an advantage over other targeted approaches such as monoclonal antibody or viral vector therapies which require that a large proportion, if not all, of the cells within a metastatic site produce the desired antigenic target or be infected by the viral vector for substantial cytotoxic effect to occur.

Based on these preclinical studies, this PSA-activated TG prodrug is currently being developed for testing in clinical trials as treatment for metastatic prostate cancer.

NOTES

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Figure Legends

Figure 1. (a) Chemical structure of thapsigargin and L12ADT (b) Chemical structure of Mu-HSSKLQ-L12ADT with site of PSA hydrolysis designated by arrow.

Figure 2. (a) Comparison of proliferative growth fraction in human prostate cancer samples compared to the human prostate cancer cell line LNCaP growing in standard media or media conditioned by human osteoblasts. Localized PCa are prostate cancers obtained from radical prostatectomy specimens (N=27); Metastatic Untreated are prostate cancers obtained from men who had not received androgen ablative therapy (N=43); Metastatic Androgen Resistant are prostate cancers obtained at warm autopsy from men who had failed androgen ablative therapy (N=132). (b) Percent apoptosis of LNCaP cells treated in vitro with vehicle or 100 nM doxorubicin, taxol, or thapsigargin in standard media (i.e. high proliferation cultures) or osteoblast conditioned media (i.e. low proliferation cultures). Cells were exposed to test agents for 48hrs then cells were fixed and stained with DAPI to assess nuclear morphology. Percent apoptosis indicates percent of counted cells (N=200) with apoptotic nuclear morphology.

Figure 3. Analysis of *in vitro* response to L12ADT (A) Fura-2 ratiometric analysis of intracellular free Ca2+ response in TSU PSA non-producing human bladder cancer cells exposed to either 250 nM TG, 250 nM L12ADT, or 10 μM Mu-HSSKLQ-L12ADT prodrug. (B) Translocation of apoptosis inducing factor from mitochondria in vehicle treated LNCaP human prostate cancer cells (left panel) to nuclei of cells treated fro 48 h with 200 nM L12ADT (right panel). (C) Kinetics of the increase in proportion of unfixed LNCaP cells positive for enzymatically active caspase 3 following exposure to 200 nM L12ADT. (D) Kinetics of the increase in proportion of unfixed LNCaP cells positive by annexin-V staining of the plasma membrane following exposure to 200 nM L12ADT. (E) Phase contrast of vehicle treated LNCaP cells. (F) Phase contrast morphology of unfixed LNCaP cells after 48 h exposure to 200 nM L12ADT. Arrows denote several cells undergoing membrane blebbing as part of their apoptotic death.

Figure 4. Cytotoxic response to continuous and episodic administration of L12ADT, and Mu-HSSKLQ-L12ADT. (A) Cell viability of PSA-producing LNCaP cells treated either continuously with 500 nM or episodically with 500 and 1000 nM Mu-HSSKLQ-L12ADT was compared to growth of untreated controls. For episodic treatment, cells were exposed to prodrug in serum containing media for 8 hrs then media removed and replaced with fresh serum containing media. Media was changed daily for indicated times. (B) Cell viability of PSA non-producing HCT-116 human colon cancer cells treated continuously with 500 nM of either L12ADT or Mu-HSSKLQ-L12ADT. were completed each day according to instructions. Standard curve were generated for each cell line to convert absorbance units to cell numbers. Each represented data point represents average of 8 wells ± the standard error and experiments were done in duplicate.

Figure 5. Pharmacokinetic analysis of plasma levels of Mu-HSSKLQ-L12ADT and L12ADT. Male mice were given a single intravenous injection of 7mg of the prodrug/kg. At various time points, blood was obtained from anesthetized mice and plasma isolated and extracted. Spiked serum samples that underwent a similar extraction procedure were used as calibration standards. Data represent average ± standard error for 3 mice at each time point.

Figure 6. (a) Response of PSA-producing LNCaP xenografts to intravenous prodrug. Data presented represent relative change in average tumor volume ± standard error of 10 animals per treatment group. Relative change in tumor volume was calculated by dividing tumor volumes measured during course of therapy by the initial tumor volume (i.e. day 0) for each individual animal. (b) Response of PSA-producing LNCaP xenografts to continuous subcutaneous infusion of Mu-HSSKLQ-L12ADT. Animals received 28 days of continuous infusion delivered by subcutaneously implanted osmotic minipump. (c) Response of PSA non-producing SN12C human renal cell xenografts to prodrug. Results represent average tumor volumes ± standard error for 10 treated animals in each experiment.

Table 1. Generality of cytotoxic response of normal and malignant cells to thapsigargin

Human Cell Type	Designation	Androgen Receptor (gene status)	Androgen Growth Responsiveness	Cellular Response to Thapsigargin (% decrease in viable cells)[a]
Prostate Cancer	LNCaP	+ (mutant)	+	88 ± 6
Prostate Cancer	MDA-PCA-2B	+ (mutant	+	79 ± 5
Prostate Cancer	LAPC-4	+ (wild type)	+	85 ± 2
Prostate Cancer	C4-2B	+ (mutant)	-	91 ± 4
Prostate Cancer	CWR22R	+ (mutant)	-	89 ± 5
Prostate Cancer	DU-145	-	-	78 ± 6
Prostate Cancer	PC-3	-	-	88 ± 3
Renal Cancer	SN12C	-	-	82 ± 6
Bladder Cancer	TSU	-	-	91 ± 4
Colon Cancer	HCT-116	-	-	90 ± 6
Medullary Thyroid Cancer	TT	-	-	82 ± 7
Breast Cancer	MCF-7	+ (wild type)	-	83 ± 2
Normal Fibroblast	WI-38	-	-	79 ± 6
Normal Osteoblast	NHO	+ (wild type)	-	81 ± 7
Normal Microvein endothelial cells	HMVEC	-	-	85 ± 6
Normal Macrovein endothelial cells	HuVec	-	-	82 ± 2
Normal Prostate epithelial cell	PREC	-	-	91 ± 4

[a]Decrease in number of viable cells after 5 day exposure to 100 nM thapsigargin expressed as a percentage of vehicle only treated cells

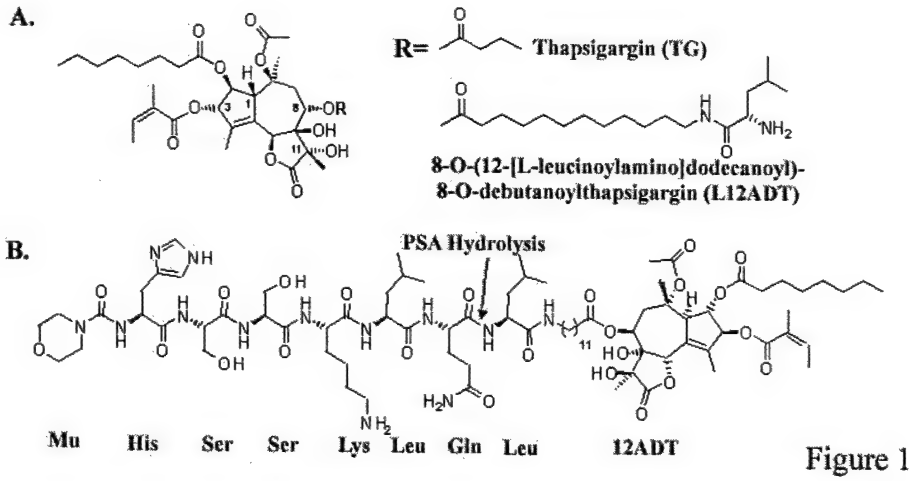

A.
R=
Thapsigargin (TG)
8-O-(12-[L-leucinoylamino]dodecanoyl)-
8-O-debutanoylthapsigargin (L12ADT)
B.
PSA Hydrolysis
Mu
His
Ser
Ser
Lys
Leu
Gln
Leu
12ADT

Figure 1

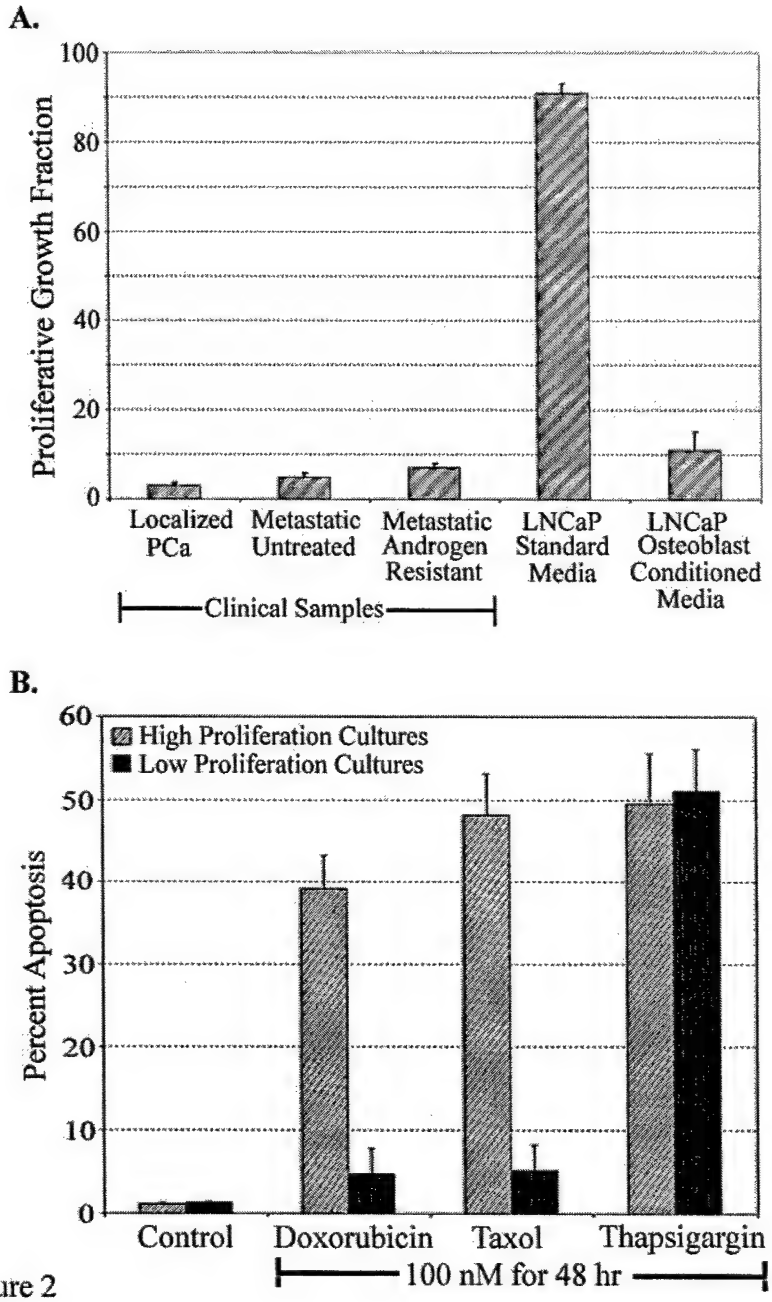

A.
Proliferative Growth Fraction
100
80
60
40
20
0
Localized PCa
Metastatic Untreated
Metastatic Androgen Resistant
LNCaP Standard Media
LNCaP Osteoblast Conditioned Media
Clinical Samples
B.
Percent Apoptosis
60
50
40
30
20
10
0
High Proliferation Cultures
Low Proliferation Cultures
Control
Doxorubicin
Taxol
Thapsigargin
100 nM for 48 hr

Figure 2

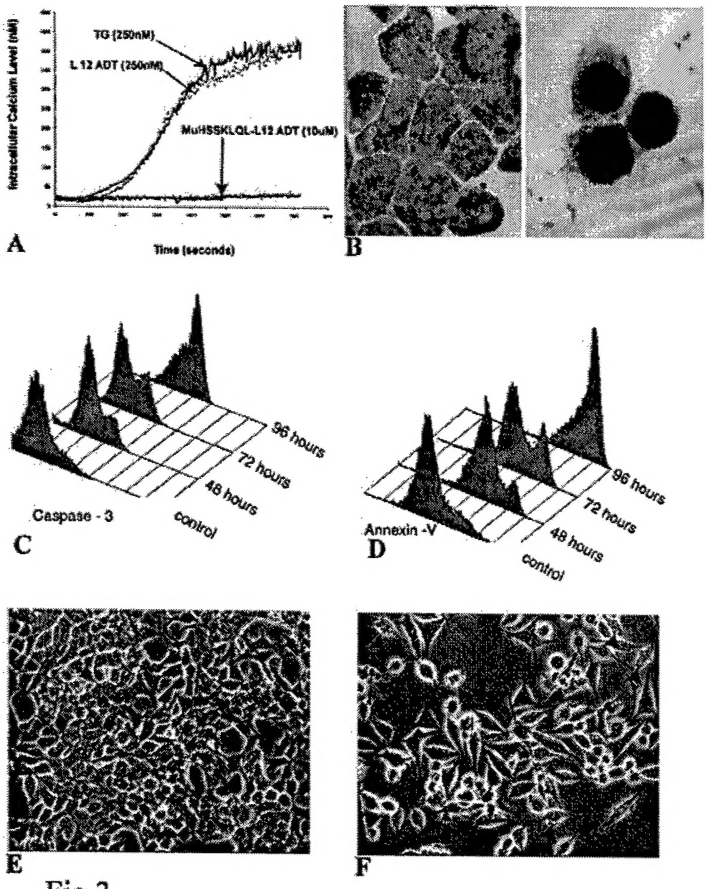

TG (250nM)
L 12 ADT (250nM)
MuHSSKLQL-L12 ADT (10uM)
Intracellular Calcium Level (nM)
Time (seconds)
A
B
96 hours
72 hours
48 hours
control
Caspase - 3
C
96 hours
72 hours
48 hours
control
Annexin -V
D
E
F

Fig 3

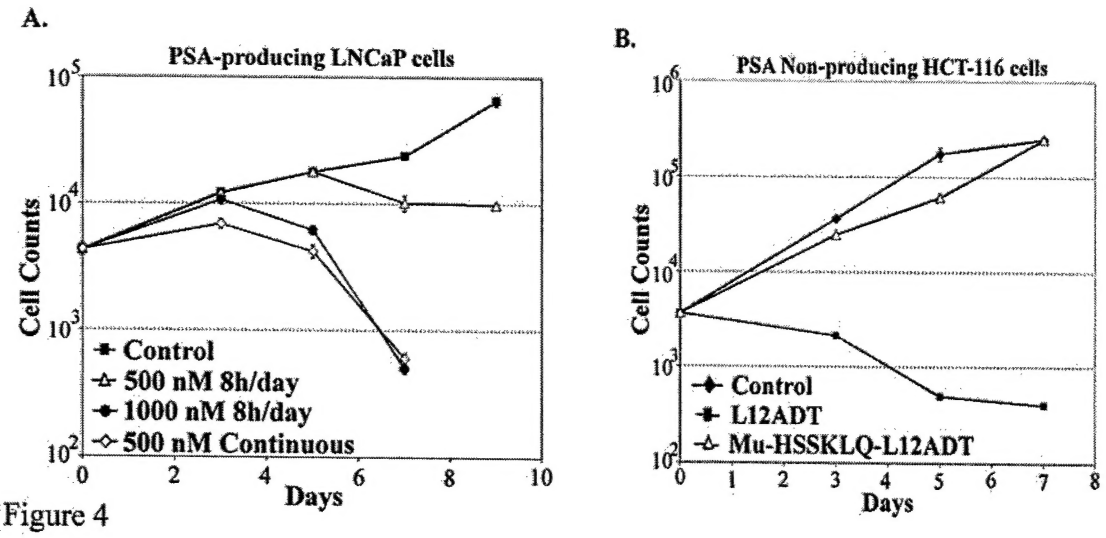

A.
PSA-producing LNCaP cells
Cell Counts
Days
Control
500 nM 8h/day
1000 nM 8h/day
500 nM Continuous
B.
PSA Non-producing HCT-116 cells
Cell Counts
Days
Control
L12ADT
Mu-HSSKLQ-L12ADT

Figure 4

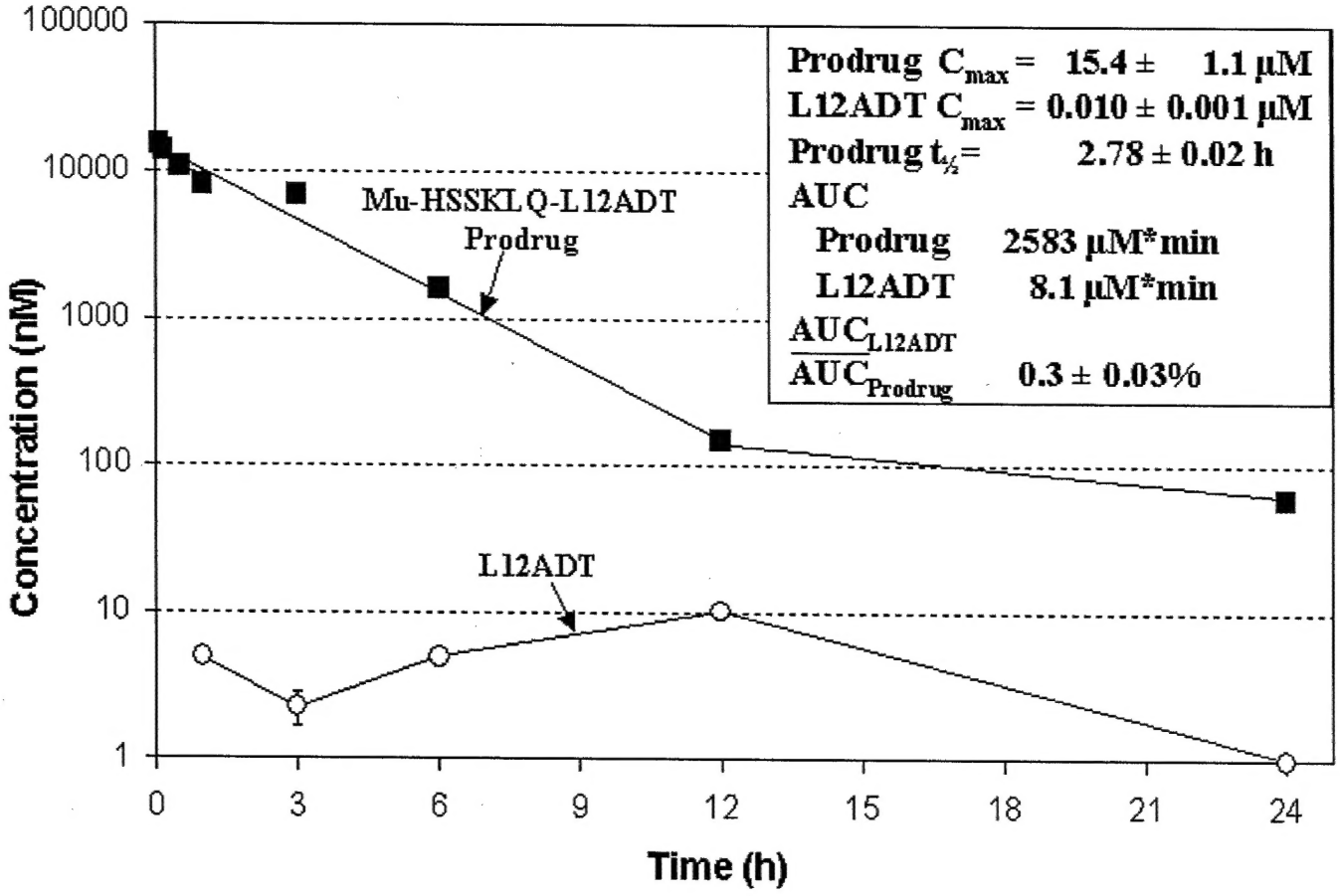
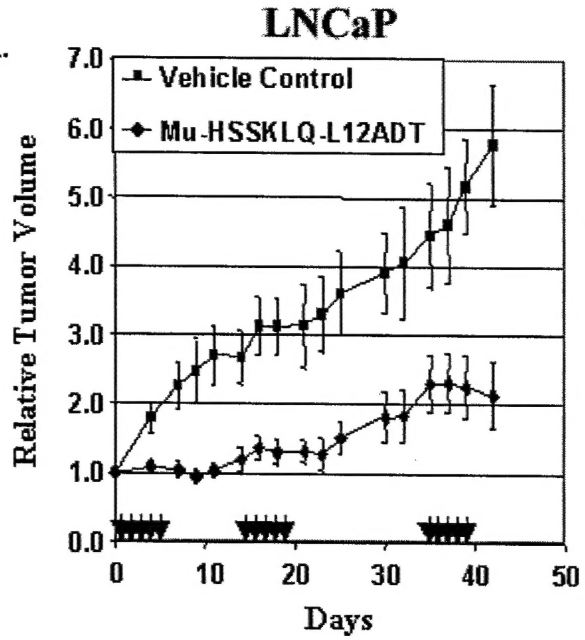
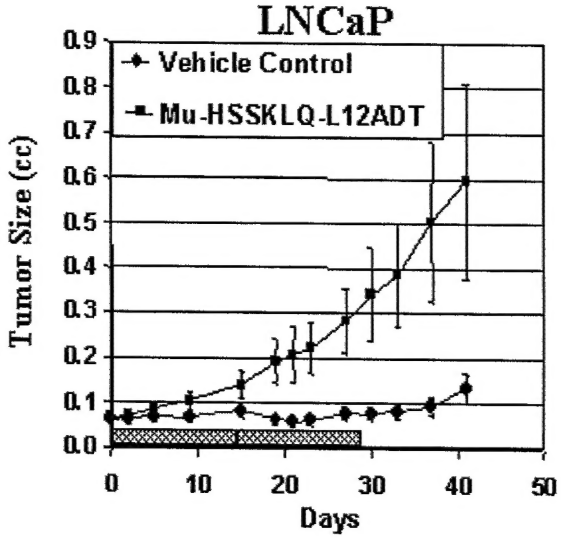

Prodrug C_{max} = 15.4 ± 1.1 µM
L12ADT C_{max} = 0.010 ± 0.001 µM
Prodrug $t_{½}$ = 2.78 ± 0.02 h
AUC
Prodrug 2583 µM*min
L12ADT 8.1 µM*min
AUC_{L12ADT} / $AUC_{Prodrug}$ 0.3 ± 0.03%
Mu-HSSKLQ-L12ADT Prodrug
L12ADT
Concentration (nM)
Time (h)
100000
10000
1000
100
10
1
0
3
6
9
12
15
18
21
24

Figure 5

A.


LNCaP
Vehicle Control
Mu-HSSKLQ-L12ADT
Relative Tumor Volume
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
Days

B.


LNCaP
Vehicle Control
Mu-HSSKLQ-L12ADT
Tumor Size (cc)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
Days

C.

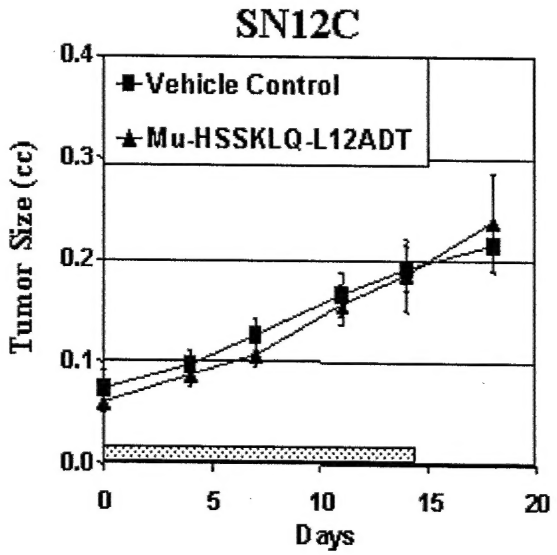

SN12C
Vehicle Control
Mu-HSSKLQ-L12ADT
Tumor Size (cc)
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
Days

Figure 6